UNIVERSIDAD COMPLUTENSE MADRID

Proyecto de Innovación y Mejora de la Calidad Docente

Convocatoria 2014

Nº de proyecto: PIMCD 2014-274

Título del proyecto: Adaptación del Laboratorio de Bioquímica y Biología Molecular I (grado de Bioquímica) a su enseñanza en inglés.

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Centro: Facultad de CC Químicas-Biológicas

Departamento Bioquímica y Biología Molecular I

INFORME FINAL DEL PROYECTO PIMCD 2014-274

1. OBJETIVOS PROPUESTOS EN LA PRESENTACIÓN DEL PROYECTO

El INGLÉS es el idioma por excelencia en las comunicaciones científicas tanto orales (congresos, conferencias) como escritas (artículos, informes científicos). Esta competencia comunicativa es por tanto imprescindible para los alumnos del grado de BIOQUÍMICA, ya que les permitirá desenvolverse con soltura en el ámbito de su profesión.

De carácter eminentemente práctico, la asignatura Laboratorio de Bioquímica y Biología Molecular I correspondiente al segundo curso del grado de Bioquímica, se prolonga durante 2 meses (108 horas presenciales, 9 créditos) con sesiones de trabajo en el laboratorio de 4 horas, lo cual permite un seguimiento cercano del alumno y de su trabajo. Esta asignatura se divide en dos turnos de modo que cada turno se compone de entre 25 y 30 alumnos.

Este escenario es ideal para la adaptación de esta asignatura al idioma inglés porque se sigue muy de cerca el trabajo experimental del alumno y su comprensión de los contenidos exigidos.

El proyecto que se presenta, pretende por tanto la utilización progresiva del inglés en la impartición de esta asignatura: el alumno se familiarizará con el uso de herramientas y contenidos de inglés científico y mejorará sus competencias lingüísticas.

Los objetivos de este proyecto son:

En cuanto al ALUMNADO:

1. Transmitir a los alumnos la IMPORTANCIA del uso del inglés científico en la redacción de artículos y en la presentación de resultados en la comunidad científica.

2. Hacer PARTÍCIPES a los alumnos del proyecto dándole las pautas necesarias para la redacción de sus resultados en inglés.

3. MOTIVAR al alumnado en la discusión oral de los resultados en inglés.

4. Aumentar las COMPETENCIAS LINGÜÍSTICAS que deben adquirir en el Grado de Bioquímica y que además pueden resultarles beneficiosas en su incorporación laboral.

Será importante que el alumno no se vea obligado sino alentado en el proceso, fomentando el uso del inglés de modo progresivo a lo largo de la asignatura.

En cuanto al PROFESORADO:

Existe cierto recelo entre los docentes a cambiar de idioma en las clases que imparten por el esfuerzo extra que supone y el miedo escénico a hablar en un idioma que no es el materno. Por ello, con este proyecto se pretende:

1. Dar los cauces necesarios para la progresiva implantación del inglés en la asignatura, comenzando con la parte escrita que suele resultar más fácil para terminar con la expresión oral que es la que suele costar más en la práctica.

2. Consolidar un GRUPO DE TRABAJO formado por profesores comprometidos con la innovación y mejora de la docencia; fomentar el trabajo en equipo permitiendo un ambiente propicio para la comunicación en inglés.

En cuanto a la TITULACIÓN:

1. Servir de prueba piloto para la posible implantación del idioma inglés en el resto de las asignaturas prácticas que deben cursar los alumnos de Grado de Bioquímica y que siguen una dinámica similar a la del Laboratorio de Bioquímica I.

2. REFORZAR la calidad de la enseñanza del Grado de Bioquímica en el Departamento de Bioquímica y Biología Molecular I de la UCM y mejorar de este modo su adaptación al Espacio Europeo de Educación Superior.

El manejo del idioma inglés es una COMPETENCIA TRANSVERSAL útil en cualquier rama de conocimiento ya que es la vía de comunicación de resultados más aceptada a nivel internacional. La realización de este proyecto permitirá MEJORAR las competencias lingüísticas del alumno y la experiencia adquirida tanto en la presentación de resultados en forma escrita como en la oral, es perfectamente extrapolable a otras ramas de conocimiento.

2. OBJETIVOS ALCANZADOS UNA VEZ FINALIZADO EL PROYECTO

Una vez realizado el proyecto, los objetivos alcanzados han sido los siguientes:

En cuanto al ALUMNADO:

1. Se ha transmitido a los alumnos la IMPORTANCIA del uso del inglés en el ámbito profesional y científico. La mayor parte del alumnado ha aceptado positivamente las tareas propuestas en inglés, aunque otro sector ha manifestado su reserva por no dominar el idioma. La idea de realizar un póster en inglés con sus resultados experimentales ha motivado a casi la mitad del grupo de estudiantes (ANEXO 7).

2. Los alumnos han tenido que presentar en inglés parte de sus resultados en forma escrita (ANEXOS 5, 6, 7).

3. En general los alumnos han adquirido mejores competencias en cuanto a inglés científico como demuestra la mejora en la evaluación del test de nivel realizado al principio y al final del curso (ANEXO 1).

En cuanto al PROFESORADO:

1. Se ha empezado a dar los primeros pasos para la implantación del inglés en la asignatura aunque sólo se ha abordado la parte lectora y escrita en detrimento de la oral.

2. Se ha formado un GRUPO DE TRABAJO que podrá en futuras convocatorias de proyectos o de la asignatura, seguir trabajando en equipo y desarrollar nuevos proyectos de innovación.

En cuanto a la TITULACIÓN:

Esta es la primera asignatura de este tipo (Laboratorio Integrado) en la que se aborda la utilización de contenidos en inglés. La continuidad del trabajo realizado serviría para REFORZAR la calidad de la enseñanza del Grado de Bioquímica en el Departamento de Bioquímica y Biología Molecular I de la UCM y mejorar de este modo su adaptación al Espacio Europeo de Educación Superior.

Dificultades encontradas:

Los objetivos que por falta de presupuesto no se realizaron tal y como se plantearon en la primera presentación del proyecto fueron:

Mejora de las competencias orales en el idioma inglés en el grupo de profesores (este objetivo quedó como voluntario para cada uno de los miembros del proyecto).

Elaboración de la PÁGINA WEB con contenidos específicos de esta materia. En su lugar se dispone de documentos y material docente que podrá ser utilizado en el futuro como modelo de trabajo.

3. METODOLOGÍA EMPLEADA EN ELPROYECTO

Para la realización de los objetivos citados anteriormente, el proyecto se ha dividido en 3 fases en las que se han realizado las siguientes actividades:

- PRIMERA FASE (antes del inicio de las clases):

- Reuniones de los cuatro profesores que forman el grupo de trabajo para fijar criterios.

-Adaptación de los contenidos existentes en el Campus Virtual al idioma inglés: guión de laboratorio, fichas resumen, problemas, procedimientos de trabajo, etc.

- Diseño del cuestionario de nivel de inglés.

- SEGUNDA FASE (durante las clases): Introducción del inglés de modo gradual.

- Cuestionario de nivel de inglés al comienzo del curso y final del mismo.

- Seminario de búsquedas de bibliografía científica en inglés (uso del PubMed).

- Utilización de material en inglés durante las explicaciones teóricas en el aula.

- Respuestas a las cuestiones del Bloque 2 de la asignatura por escrito en inglés en el cuaderno de laboratorio del alumno.

- Elaboración por los alumnos en inglés de algunas partes de los informes de resultados de la asignatura y/o de un póster científico.

- TERCERA FASE (tras finalizar las clases presenciales):

- Reunión para la evaluación final del Proyecto y elaboración de los resultados finales.

- Evaluación de la prueba de nivel de inglés. En la prueba inicial se pregunta a los alumnos sobre el nivel de inglés que consideran tener.

El test consiste en tres bloques de preguntas cortas. El bloque A es de comprensión lectora: los alumnos deben responder a preguntas cortas según la información del texto científico que se les ofrece. El bloque B consiste en 5 frases con errores gramaticales (concordancia de número, vocabulario, etc.). El bloque C consiste en la traducción al inglés de frases con contenido científico.

Cada ítem se puntúa como 1 (Correcto) o 0 (inválido) excepto en el tercer bloque (0, 0.5 ó 1). El número de ítems acertados (máximo 13 en el cuestionario inicial y 15 en el final) se promedia sobre 10 para dar la nota final en una escala de 0 a 10. Los alumnos dispusieron de unos 30 minutos para realizar el test. Los resultados se procesaron mediante los programas de Excel y GraphPad 5 Prism.

4. RECURSOS

Recursos humanos:

Todos los recursos humanos pertenecen al Departamento de Bioquímica y Biología Molecular I de la UCM pero están adscritos a distintas facultades: Químicas (1) o Biológicas (2):

La Profesora Titular Ana Saborido Modia (AS, 1)

El Profesor Titular Miguel Arroyo Sánchez (MA, 2)

La Profesora Titular Maria José Feito Castellano (MJF, 2)

La Profesora Titular Juana María Navarro Llorens (JMN, 1).

Recursos materiales:

Este proyecto no ha tenido ninguna fuente de financiación. Los gastos derivados del desarrollo del proyecto han sido minimizados, utilizando preferentemente los medios que el Departamento pone a disposición de los profesores y los voluntariamente cedidos por estos últimos.

Material fungible: paquetes de folios; cartuchos de impresora; lápices de memoria; varios de papelería. Se ha utilizado la fotocopiadora del Departamento y material de prácticas. Parte del papel procede de material perteneciente a los grupos de investigación en los que están integrados los componentes de este proyecto. Las memorias USB las aportaron de modo voluntario los profesores de este proyecto.

Red informática de la UCM

Impresoras (particulares y del Departamento), lápices de memoria (particulares).

Materiales consumibles (particular y del Departamento)

Cámara fotográfica (particular)

Ordenadores y cañón (particular o del Departamento).

La presentación e impresión de los poster corrió a cargo de los alumnos; para minimizar su gasto se decidió no usar un tamaño superior a DinA3. La exposición pública de los posters a mayor tamaño en las paredes del laboratorio no ha sido posible debido a la nula dotación económica del presente proyecto.

Material inventariable: Se sustituyó la propuesta de diccionario físico en el laboratorio con el acceso *on line* a los diccionarios gratuitos. Un problema fue la lentitud del acceso a internet de los laboratorios de prácticas y la antigüedad de los equipos informáticos.

5. DESARROLLO DE LAS ACTIVIDADES

5.1. PRIMERA FASE:

El día 27 de junio de 2014 se nos comunicó vía mail la concesión de este proyecto en la segunda fase sin dotación económica. Se realizó una reunión durante la primera semana de julio con los 4 componentes del proyecto, en la que se trataron varios temas:

a) Se procedió a repartir las tareas para completar la primera fase del proyecto:

AS. Elaboración de un glosario (ANEXO 4) para cada unidad con los términos científicos más importantes y de uso frecuente para el alumno. Adaptación al inglés de las cuestiones planteadas a los alumnos en el Bloque II de la asignatura.

MA. Adaptación al inglés de las fichas de trabajo más relevantes para el alumnado.

MJF. Elaboración de los cuestionarios de nivel de inglés que se darán a los alumnos. Adaptación al inglés de las cuestiones planteadas a los alumnos en el Bloque II de la asignatura.

JMN. Adaptación del guión al inglés y elaboración de fichas con los procedimientos de trabajo para cada parte de la asignatura.

b) El plan de trabajo y los objetivos propuestos inicialmente en la petición de este proyecto han sido reajustados debido a la ausencia de dotación económica.

En concreto: - la mejora de las competencias orales en inglés científico de los docentes participantes (con clases puntuales para mejorar la expresión oral) se dejó a cargo de cada uno de los integrantes.

- Se prescindió de la compra de diccionarios y se remitió al alumno al uso del diccionario *on line*, aunque los laboratorios de bioquímica no disponen más que de tres ordenadores antiguos con una conexión muy lenta a internet para todos los alumnos.

Durante los meses de julio y agosto de 2014, se llevaron a cabo las tareas acordadas en la reunión del inicio de lanzamiento del proyecto en junio.

El 2 de septiembre se realizó otra reunión para revisar el material en inglés elaborado hasta el momento y concretar un calendario para terminar el material pendiente y completar así los objetivos.

Con posterioridad, el 11 de septiembre los miembros del equipo acordaron lo siguiente:

- Se fija el Test en inglés que se entregará al alumno al inicio y al final de la asignatura (ANEXO 2). En este Test se pedirá a los alumnos que contesten a unas preguntas después de leer un párrafo científico. También se les pedirá que traduzcan frases prototipo en lenguaje científico.

- Se fijan las exigencias mínimas en inglés que se pedirán a los alumnos:

Bloque I: se les entregará el glosario en inglés y en las clases se incluirán vídeos en inglés sobre las materias a tratar cuando proceda.

Bloque II: las cuestiones en este apartado se plantean en inglés, las respuestas deberán ser respondidas en dicho idioma por los alumnos y deberán reflejarse en el cuaderno de laboratorio

para su posterior corrección. En el campus virtual (CV) el alumno dispondrá de la versión en español de estas cuestiones para aquél que requiera su utilización, pero la respuesta deberá ser en inglés.

Se continuará poniendo vídeos en inglés cuando proceda. Las presentaciones ofrecidas a los alumnos incluirán términos en inglés para profundizar en el lenguaje específico de cada unidad.

Bloque III: el informe de resultados de carácter individual que los alumnos entregarán para su posterior evaluación, incluirá un resumen en inglés.

Bloque IV: el informe de resultados individual a entregar en este bloque, incluirá el resumen y las conclusiones en inglés.

Para todos los bloques, el alumno tendrá a su disposición fichas en inglés sobre aspectos importantes para su aprendizaje: e.g. Uso de la bibliografía.

Finalmente y de modo voluntario, se ofrecerá la posibilidad de realizar un póster en inglés con los resultados del bloque IV, individualmente o en equipo de no más de 4 personas. La entrega de este póster se realizará el 12 de ENERO en formato DIN A3 y en versión electrónica (ppt, pdf, etc.). Los pósters se expondrán en el laboratorio de prácticas para que quede constancia y sirva de referencia a futuras promociones.

Durante el mes de septiembre de 2014 se actualizó el CV y se incorporaron los contenidos elaborados en inglés en una carpeta especial: guión, fichas experimentales para los bloques I-II, un glosario científico, fichas de cómo usar el pHmetro y de búsqueda bibliográfica y consejos para mejorar el inglés científico (Figura 1 de resultados). Además se incluyó un documento con las cuestiones del bloque II en español, por si algún estudiante tenía problemas en entender el enunciado de las cuestiones que aparecían en inglés en el guión.

5.2. SEGUNDA FASE

5.2.1. Prueba de nivel/ Cuestionario inicial

El primer día de clase se les entrega un test de nivel de inglés a rellenar (ANEXO 2). Los datos se analizan según se detalla en resultados. Esta prueba nos facilita comprobar el nivel previo de los alumnos. Se deduce de los resultados que el nivel es de tipo intermedio y con mejor capacidad lectora (compresora) que de redacción (escrita).

5.2.2. Uso del PubMed.

El 24 de octubre y el 3 de noviembre se realizaron búsquedas bibliográficas en Bases de Datos Internacionales para que tomaran contacto con el manejo del inglés a nivel científico en un entorno informático; de esta forma se buscaron términos como “lysozyme” y “egg white” usando la herramienta PubMed en una de las aulas de ordenadores de la Facultad de CC Químicas. Los alumnos trabajaron en formando equipos de dos integrantes.

5.2.3. Utilización de vídeos en inglés

Durante la realización de los bloques I, II y III de la asignatura (durante septiembre, octubre y primeros de noviembre), se acompañaron las explicaciones de cada jornada con vídeos en inglés (Tabla 1).

Siempre que se pudo se utilizaron subtítulos en inglés para ayudar al alumnado al seguimiento de los vídeos.

VIDEOS EN INGLES DEL BLOQUE 1:	Uso de las micropipetas: video de la Universidad de Leicester http://www2.le.ac.uk/projects/oer/oers/genetics/using-a-micropipette Cómo usar un colorímetro: https://www.youtube.com/watch?v=QdufRwbkeKo
VIDEOS EN INGLES DEL BLOQUE 2:	Fundamentos de la cromatografía de exclusión molecular: http://www.austincc.edu/biocr/1406/laba/sec/ Cómo montar una columna; https://www.youtube.com/watch?v=R491QOnkmCs Electroforesis en PAGE.SDS https://www.youtube.com/watch?v=EDi_n_0NiF4 Electroforesis de DNA en geles de agarosa https://www.youtube.com/watch?v=96Gss_YVo84 https://www.youtube.com/watch?v=U2-5ukpKg_Q
VIDEOS EN INGLES DEL BLOQUE 3:	Valoración de la concentración de proteína por Bradford: https://www.youtube.com/watch?v=vfY3mVOlGBU

Tabla 1: relación de vídeos presentados a los alumnos en los bloques I, II y III.

5.2.4. Realización de actividades en inglés

En el guión de laboratorio, al final de cada práctica de los bloques I y II aparecen cuestiones complementarias a responder por los alumnos. Las correspondientes al bloque II se han incluido en inglés (ANEXO 3A). En el ANEXO 3B se recogen extractos del cuaderno de laboratorio de algunos alumnos con la resolución de estos problemas. De igual modo, en el ANEXO 5 y en el ANEXO 6 se recogen extractos de los informes entregados por los alumnos correspondientes a los bloques III y IV con el ABSTRACT en inglés.

5.2.5. Realización del Prueba de nivel /cuestionario final

La prueba final es igual que la inicial excepto en que se eliminó la valoración inicial personal del idioma y en que se han añadido dos ítems más en el apartado C relativos a los bloques III y IV. Los días 3 y 4 de diciembre se rellenaron los cuestionarios finales cuyos resultados se recogen en las figuras 6, 7 y 8 y en la tabla 2 (ANEXO 1).

5.2.6. Presentación de pósters

Por último, en el material adicional presentado (ANEXO 7) se recogen las presentaciones tipo póster realizadas por los alumnos correspondientes a los resultados del bloque IV de la asignatura: en total, 9 pósters realizados por 27 alumnos de un total de 51 alumnos.

Estas presentaciones en formato A3 se han colocado en los laboratorios donde los alumnos continuarán realizando prácticas para que quede vigente el trabajo realizado y como modelo para futuras convocatorias de la asignatura.

5.3. TERCERA FASE:

Durante el mes de diciembre se realizó una evaluación final del proyecto y se discutieron los datos de las evaluaciones realizadas. Las principales conclusiones del proyecto se recogen en el ANEXO 1.

6. ANEXOS.

ANEXO 1: Resultados y productos.

ANEXO 2: Test inicial y final de inglés para los alumnos.

ANEXO 3A: Cuestiones bloque II.

3B: Ejemplos de cuadernos de alumnos con las cuestiones resueltas

ANEXO 4: Glosario de vocabulario científico

ANEXO 5: Informe 1, ejemplos de resúmenes elaborados por los alumnos en inglés

ANEXO 6: Ejemplos de partes de los informes 2 elaborados por los alumnos en inglés

ANEXO 7. Pósters elaborados por los alumnos

ANEXO 1: RESULTADOS Y PRODUCTOS

En el campus virtual se añade una carpeta de contenidos SCIENTIFIC ENGLISH disponible para todo el alumnado como se ve en la Figura 1.

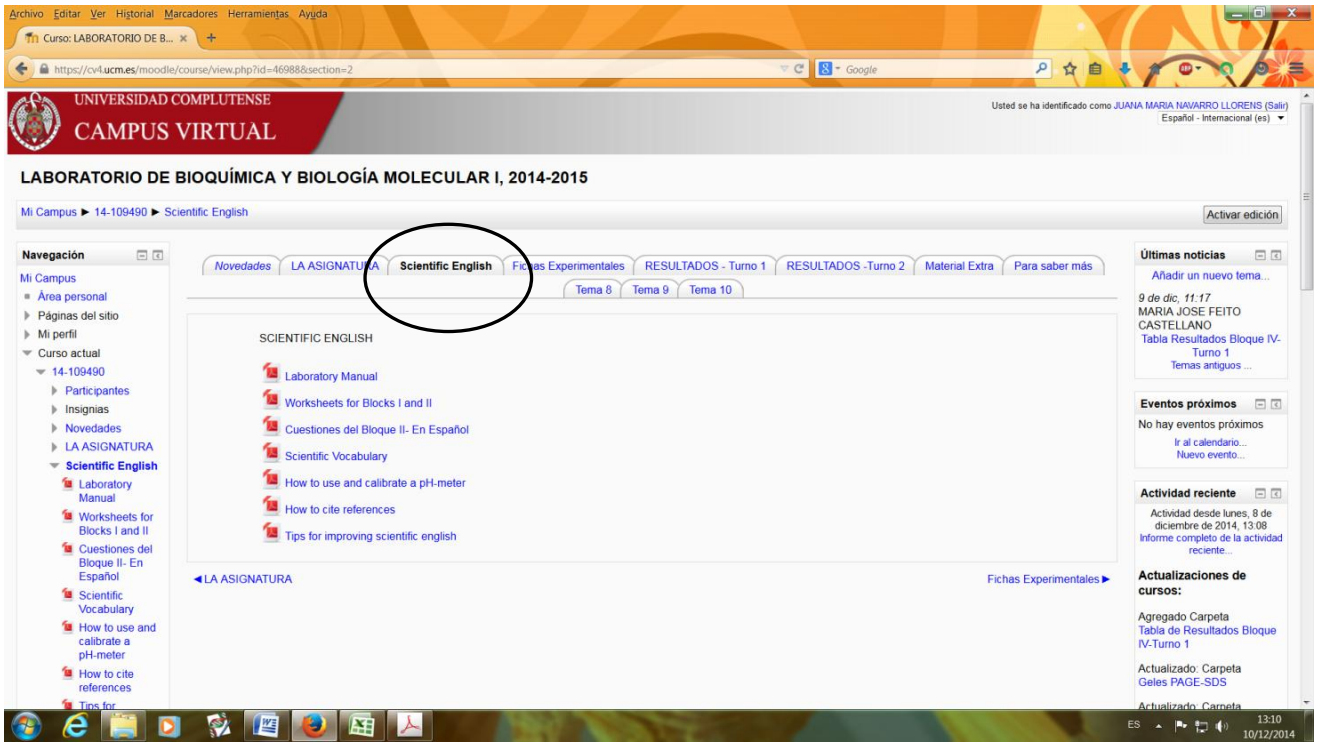


Figura 1. Vista del Campus Virtual de la asignatura. La carpeta Scientific English permite a los alumnos acceder a los diferentes materiales elaborados para ellos.

Antes de la realización del cuestionario, se les pregunta a los alumnos qué nivel de inglés valoran ellos que tienen (Básico=1 pto; Intermedio = 2 ptos, Avanzado = 3 ptos) tanto en el idioma leído, hablado y/o escrito. El nivel mínimo es un 3 (nivel básico en los 3) y el máximo un 9 (avanzado en los 3). En la Figura 2 se recoge la valoración personal del nivel de inglés y como se puede apreciar, mayoritariamente, 50%

del alumnado, considera tener un nivel intermedio, un 25% un nivel básico y otro 25% por encima de la media (6.0).

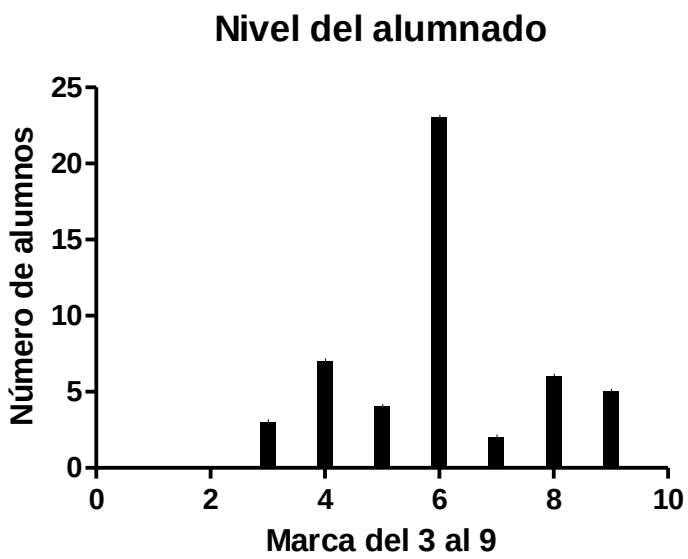


Figura 2. Valoración individual del alumnado de su nivel de inglés (3 indica básico y 9 avanzado). Total de alumnos = 50 (un alumno dejó este apartado en blanco).

Los resultados del cuestionario/prueba de nivel inicial se recogen en la figura 3, 4 y 5 y en la tabla 2.

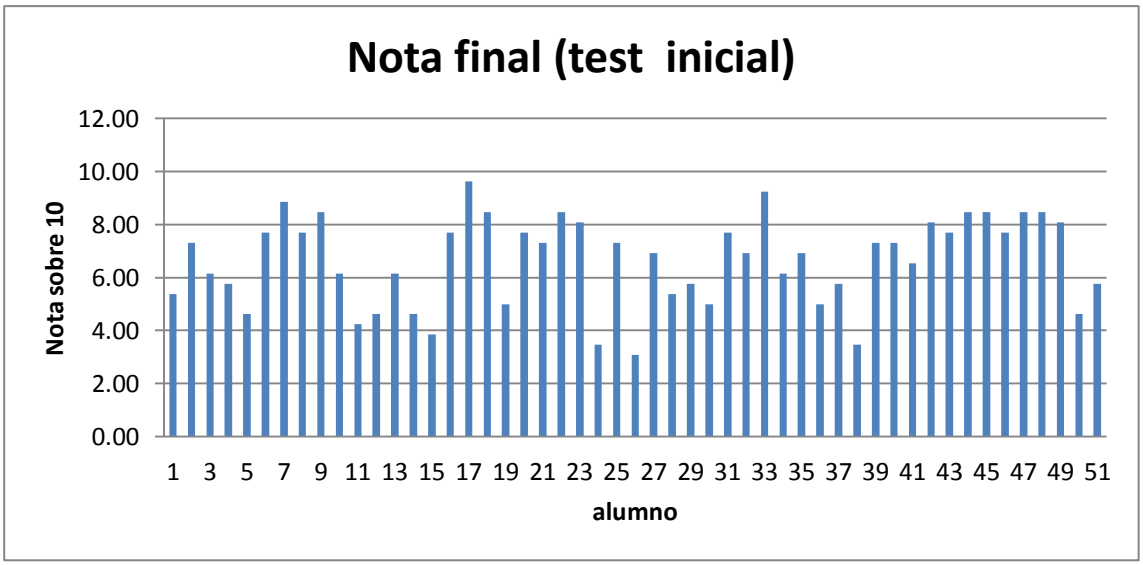


Figura 3. Nota final por alumno del cuestionario/prueba de nivel de inglés inicial.

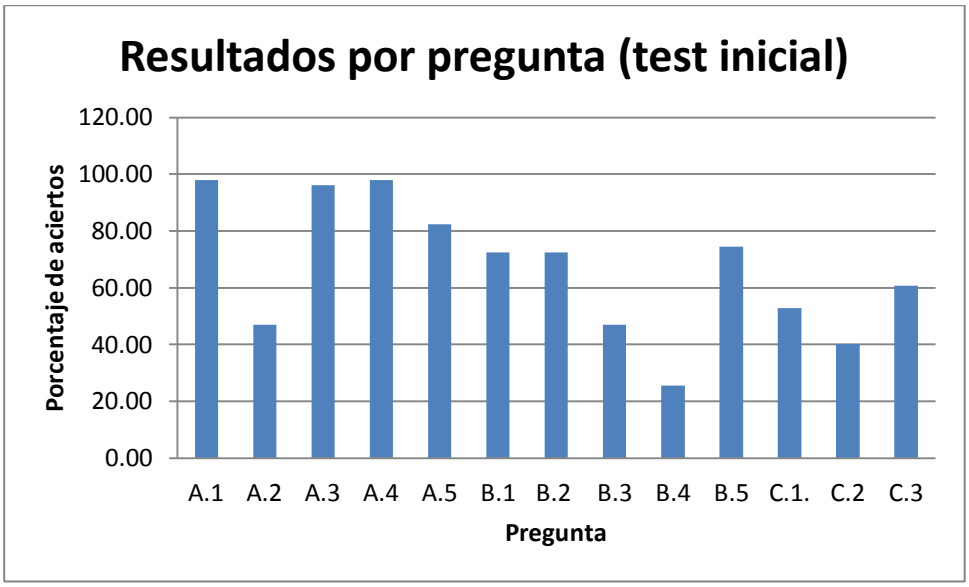


Figura 4. Porcentaje de aciertos por pregunta en el test inicial.

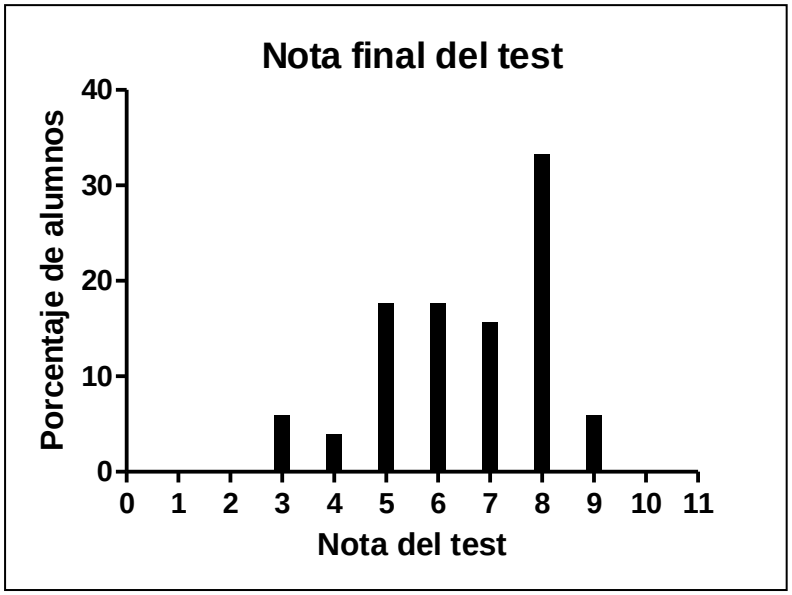


Figura 5. Nota final del cuestionario 1 por porcentaje de alumnado.

Sobre un máximo de 10, la nota media de la muestra de alumnos fue de 6.6 en el cuestionario inicial. La pregunta A que trata sobre la compresión de un texto alcanza casi un 85% de aciertos (Tabla 3) mientras que la pregunta B (corrección de errores básicos) y la C (traducción del español a inglés) se quedaron en torno a 58 y 51 % respectivamente de aciertos (Tabla 3) .

La nota media alcanzada en el test inicial, un 6.6, supone un buen punto de partida para trabajar con los alumnos. Además, queda manifiesto con este Test que la comprensión lectora es mucho mejor que la expresión escrita. Queda patente que un 25% del alumnado con un nivel básico de conocimientos, se

encuentra con problemas en el uso de este idioma. En el abordaje que hemos hecho en este proyecto hemos tenido en cuenta estos problemas. Es por ello que por ejemplo, se han traducido las cuestiones del bloque II al español (Figura 1) y enlazado en el Campus Virtual. También se ha potenciado el trabajo en equipo (como en la elaboración de pósters) y se ha limitado la escritura del inglés al "abstract" y las conclusiones tal y como se viene pidiendo actualmente en la memoria del Trabajo de Fin de Máster.

El cuestionario final es exactamente igual que el inicial con la salvedad de eliminar la valoración inicial personal del idioma y que se han añadido dos ítems más en el apartado C relativos al bloque III y IV. Los resultados se recogen en las figuras 6, 7 y 8 y en la Tabla 3.

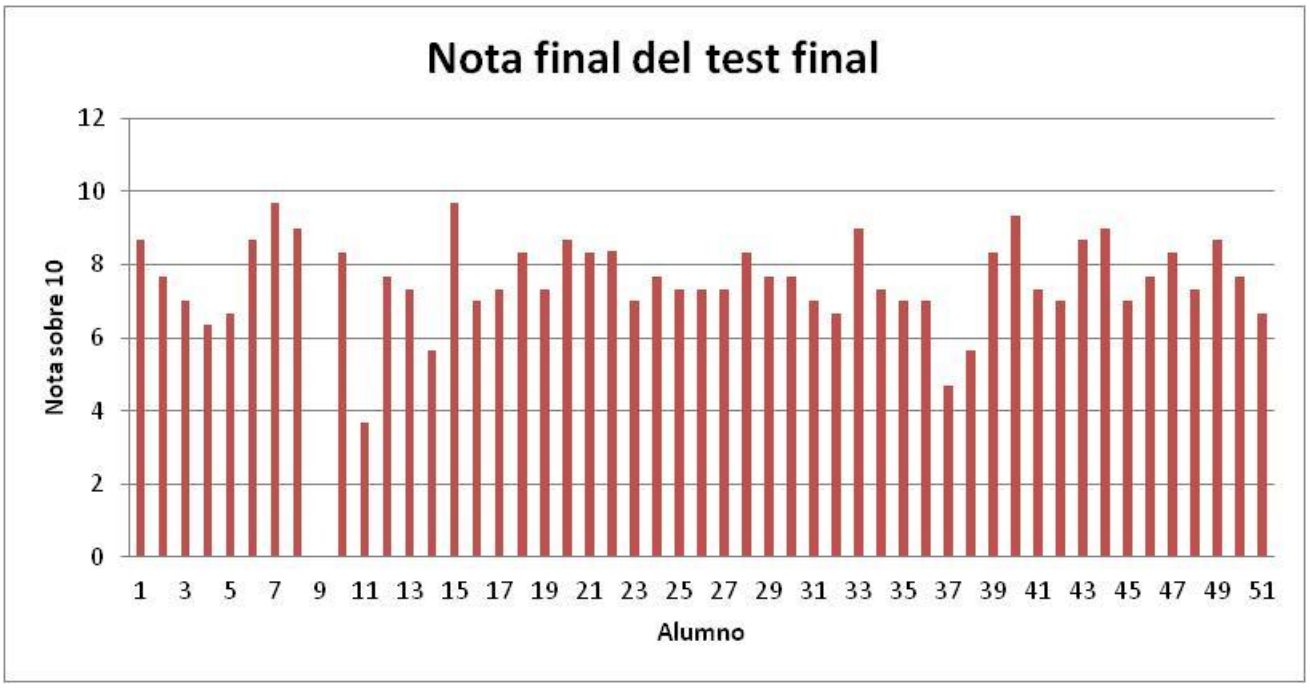


Figura 6. Nota final por alumno del cuestionario final. Ese día el alumno 9 no estuvo.

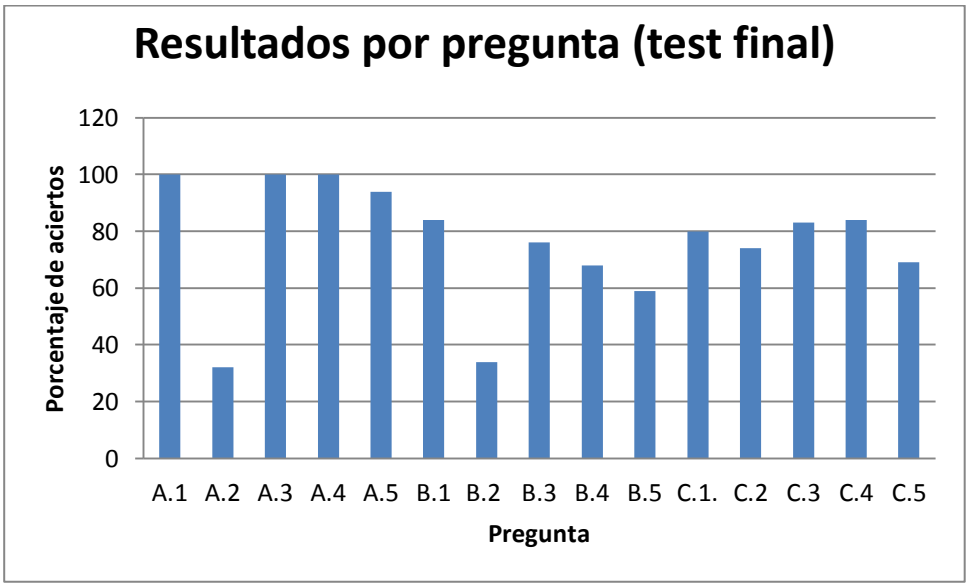


Figura 7. Porcentaje de aciertos por pregunta en el test final.

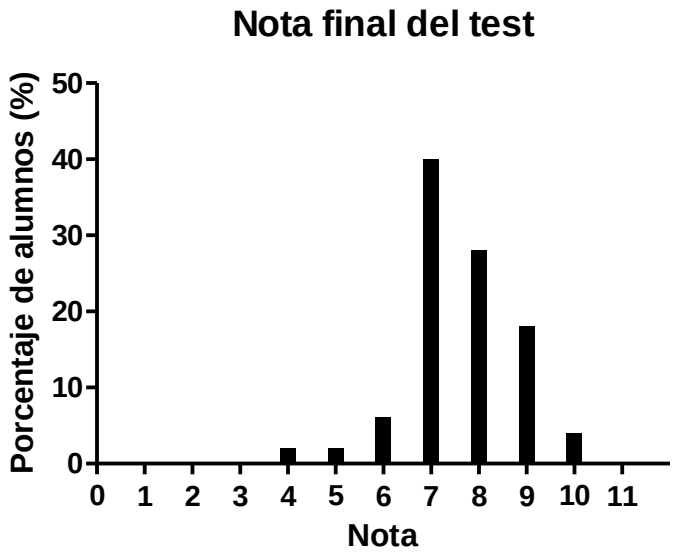


Figura 8. Nota final del cuestionario 2 por porcentaje de alumnado.

Sobre un máximo de 10, la nota media de la muestra de alumnos fue de 7.6 en el cuestionario final.), un punto más que en el cuestionario inicial. La comprensión lectora (bloque A) se mantiene en el mismo porcentaje, un 85 % mientras que la detección de errores básicos (bloque B) aumenta ligeramente del 58 al 64 % (tabla 3) y el bloque C (traducción del español a inglés) pasa del 51 al 78 % de aciertos. (Tabla 3). Este resultado es previsible ya que no se ha enseñado gramática inglesa a los alumnos si no que se ha potenciado la capacidad de redactar de modo científico. Por tanto podemos concluir que se ha producido en general una mejora sobre en las habilidades traductoras de los estudiantes durante la realización de este proyecto.

Tabla3. Porcentaje total de aciertos por pregunta en los cuestionarios de inglés

	PORCENTAJE DE ACIERTOS TEST INICIAL	**PORCENTAJE DE ACIERTOS TEST FINAL**
Pregunta A	84.4 %	85.2 %
Pregunta B	58.4 %	64.2 %
Pregunta C	51.3 %	78.0 %

En los anexos 4, 6 y 7 se recogen algunos ejemplos del trabajo en inglés realizado por los alumnos a lo largo del proyecto.

ANEXO 2 CUESTIONARIOS INICIAL Y FINAL

Level Test Biochemistry and Molecular Biology Laboratory I

Scientific English Level

Name and Surname:

Level of English:	Reading	Writing	Speaking
Elemental/Basic			
Intermediate			
Advanced			

Level of knowledge of other foreign languages:

A) Read the text and answer (in English) the questions:

This laboratory class combines molecular visualization and laboratory experimentation to teach the structure of the immunoglobulins (Ig). In the first part of the class, the three-dimensional structures of the human IgG and IgM molecules available through the RCSB PDB database are visualized using freely available software. In the second part, IgG and IgM are studied using electrophoretic methods. Through SDS-PAGE gel analysis under reducing conditions, the students determine the number and molecular masses of the polypeptide chains, while through SDS-PAGE gel under nonreducing conditions, the students assess the oligomerization of these Ig molecules. The aims of this class are to expand upon the knowledge and understanding of the Ig structure that the students have gained from classroom lectures. The combination of this molecular visualization of the Ig molecules and the SDS-PAGE experimentation ensures variety in the teaching techniques, while the implication of the Ig molecules in human disease promotes interest for biomedical students. © 2014 by The International Union of Biochemistry and Molecular Biology, 42(2):152–159, 2014.

a) What kind of biological molecules are studied in this laboratory?

b) What kind of experimental techniques are used?

c) Which information is provided on the use of SDS-PAGE gel under non-reducing conditions?

d) Which information is provided on the use of SDS-PAGE gel under reducing conditions?

e) Why is it important to study the Ig molecules?

B) Find the mistakes and correct them (one or more in each sentence).

- The pH effected the kinetic behavior.

- This value is rare compared to that of the mean of the class.

- Using the maxima wavelength gives us the best results.

- Data presented is the average of at least three experiments

- These experiments does not require any expensive equipments

C) Translate the next sentences into English.

- Las muestras se analizaron por PAGE-SDS.

- La curva de calibrado se construyó usando BSA como patrón.

- Para calibrar el espectrofotómetro necesitamos un tubo de referencia.

Scientific English Level 2014

Biochemistry and Molecular Biology Laboratory

Name and Surname:

A) Read the text and answer (in English) the questions:

This laboratory class combines molecular visualization and laboratory experimentation to teach the structure of the immunoglobulins (Ig). In the first part of the class, the three-dimensional structures of the human IgG and IgM molecules available through the RCSB PDB database are visualized using freely available software. In the second part, IgG and IgM are studied using electrophoretic methods. Through SDS-PAGE gel analysis under reducing conditions, the students determine the number and molecular masses of the polypeptide chains, while through SDS-PAGE gel under nonreducing conditions, the students assess the oligomerization of these Ig molecules. The aims of this class are to expand upon the knowledge and understanding of the Ig structure that the students have gained from classroom lectures. The combination of this molecular visualization of the Ig molecules and the SDS-PAGE experimentation ensures variety in the teaching techniques, while the implication of the Ig molecules in human disease promotes interest for biomedical students.

a) What kind of biological molecules are studied in this laboratory?

b) What kind of experimental techniques are used?

c) Which information is provided on the use of SDS-PAGE gel under non-reducing conditions?

d) Which information is provided on the use of SDS-PAGE gel under reducing conditions?

e) Why is it important to study the Ig molecules?

B) Find the mistakes and correct them (one or more in each sentence).

- The pH effected the kinetic behavior.

- This value is rare compared to that of the main of the class.

- Using the maxima wavelength gives us the best results.

- Data presented is the average of at least three experiments

- These experiments does not require any expensive equipments.

C) Translate the next sentences into English.

- Las muestras se analizaron por PAGE-SDS.

- La curva de calibrado se construyó usando BSA como patrón.

- Para calibrar el espectrofotómetro necesitamos un tubo de referencia.

- La figura muestra el efecto de la temperatura en los datos cinéticos de la β-glucosidasa.

- El objetivo de este estudio es la propuesta de un método de aislamiento para la lisozima.

ANEXO 3A: CUESTIONES DEL BLOQUE 2

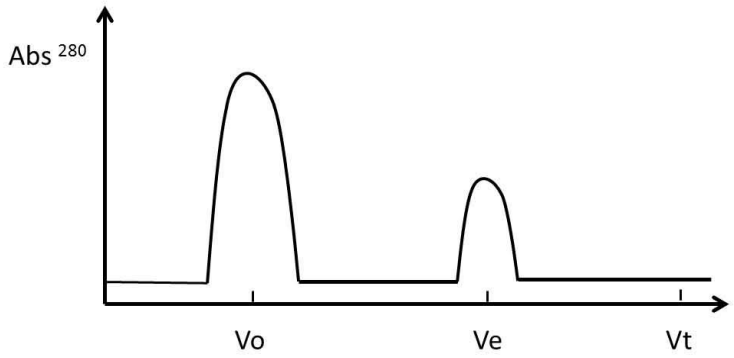
1

BLOCK 2: BASIC TECHNIQUES IN MACROMOLECULE RESEARCH

2.1. DETERMINATION OF PROTEIN MOLECULAR MASS BY SIZE-EXCLUSION CHROMATOGRAPHY

1- *[Protein desalting]*. A protein (molecular mass 240 000 Da) dissolved in 1 M potassium phosphate buffer, pH 7.5, was loaded on a Sephadex G-25 column (fractionation range for peptides and globular proteins 1000 - 5000 Da), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.5. This equilibration buffer was also used for the elution step. In which volume will you expect to elute the protein? What will be the molarity of the phosphate buffer in which the protein elutes?

2- A mixture containing three proteins was loaded on a Sephadex G-150 column (fractionation range for globular proteins 5 000 - 300 000 Da). The Figure shows the chromatographic elution profile. Why are there only two peaks in the elution profile? What techniques could you use to determine the protein composition of each peak? How could you try to separate the three proteins?



3- The molecular mass of an enzyme isolated from *Acinetobacter sp.* was determined by size exclusion chromatography. The following Kav values were obtained for standard proteins of known molecular mass (see the Table). Estimate the molecular mass of the bacterial enzyme, knowing that its Kav value is 0.67

Protein	Molecular Mass (kDa)	Kav
Cytochrome c	12	0.840
Lysozyme	14	0.790
Ovalbumin	45	0.550
Bovine serum albumin	66	0.440
Alcohol dehydrogenase	160	0.260
Pyruvate kinase	235	0.156

4- *[Determination of the number of subunits]*. A protein was purified to homogeneity. By size exclusion chromatography its molecular mass was estimated to be 60 kDa in the absence of urea, and 30 kDa when the chromatography was performed in the same buffer containing 6 M urea. When the chromatography was repeated in the presence of 6 M urea and 10 mM β-mercaptoethanol, a single peak corresponding to 15 kDa was detected. Describe the molecular structure of the protein.

2.2. DETERMINATION OF PROTEIN MOLECULAR MASS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

1- Calculate the volume of each stock solution needed to prepare a 10% polyacrylamide-resolving gel (see the Preparation Table for SDS-PAGE gels). How could you prepare a 5-15% polyacrylamide gradient-slab gel?

2- Consider that there were a 15%- and a 7%-polyacrylamide SDS-PAGE gel in the laboratory, which one would you employ to identify a protein of 15 kDa? And if the molecular mass of the protein were 150 kDa?

3- You want to analyze by SDS-PAGE electrophoresis a sample with a protein concentration of 35 mg/ml. For this purpose, an aliquot of the sample is diluted 20-fold with distilled water to a final volume of 500 µl. Then, 30 µl of the 1/20 dilution are mixed with 30 µl of sample buffer (2X), and the mixture is heated for 5 min at 90 °C. Finally, 10 µl and 20 µl of the last preparation are loaded into the gel wells. (a) Describe how you would prepare the required 1/20 dilution, if you had in the laboratory micropipettes for pipetting volumes of 2-20 µl, 20-200 µl and 200-1000 µl. (b) Calculate how many micrograms of protein are loaded into each gel well.

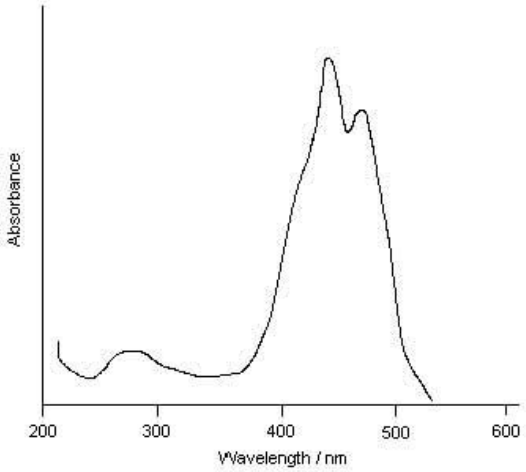
4- After an ion-exchange chromatography, a preparation containing 25 mg of protein per milliliter was obtained. To establish the purity of this preparation by SDS-PAGE, you are required to prepare two samples containing 5 µg and 10 µg of protein, respectively. Remember that the maximum loading volume per gel well is 20 microliters and that the sample loading buffer is prepared at double concentration (2X). (a) How much will you dilute the initial preparation with distilled water? (b) Describe the preparation of the two samples that must be loaded in the wells (including sample buffer).

5- You are required to load into an SDS-PAGE gel 4 µg of a sample with a protein concentration of 0.8 mg/ml. If the sample loading buffer is three times more concentrated (3X) than the final desired concentration, what volume of protein sample, distilled water and sample loading buffer (3X) should be mixed in order to load a total volume of 15 µl in the well?

6- (a) Sketch the expected elution profile (absorbance at 280 nm *vs* elution volume) for a size exclusion chromatography of a protein mixture containing cytochrome c (12 kDa) and nucleoside diphosphokinase (100 kDa, 6 subunits); identify the peaks in the profile. (b) Sketch the results that would be obtained with the same mixture of proteins if analyzed by SDS-PAGE electrophoresis; indicate the migration direction and identify each band.

2.3. CHARACTERIZATION OF PROTEINS BY SPECTROPHOTOMETRY

1- Indicate in the following spectrum: (a) The wavelength at what the peptide bond absorbs (b) Where do the aromatic amino acid side-chains absorb? Which of the aromatic amino acids may produce the peak? (c) If the protein contains a chromophore, where will it absorb? (d) Assume that you are carrying out a purification protocol and that you have to detect specifically the presence of this protein, at what wavelength would you measure it? Why? (e) At what wavelength would you measure, if you want to know the total protein content in a sample?



2- Explain the reasons of the changes observed in the spectrum of hemoglobin.

3- The extinction coefficient of a protein can be accurately determined by absorbance and dry weight measurements. A solution containing about 2 mg/ml of protein was filtered through a Millipore filter, and its absorbance at 280 nm was 0.43, when using 1 cm-pathlength quartz cuvettes. Three aliquots of 3 ml of this solution were dialyzed overnight against distilled water. Thereafter, the aliquots were pipetted into preweighed tubes and dried at 90 °C and then to 108 °C until they reached constant weight. After this, the amount of protein determined for each aliquot was: 5.6574 mg, 5.5847 mg and 5.6863 mg, respectively. Calculate the specific extinction coefficient ($E^{0.1\%}$, $(mg/ml)^{-1}$ cm^{-1}) and the molar extinction coefficient (ε, M^{-1} cm^{-1}) at 280 nm for the protein (molecular mass 12 024 Da).

4- A solution containing NAD^+ and NADH shows absorbances of 0.311 and 1.2 at 340 nm and 260 nm, respectively, in a 1 cm-pathlength cuvette. Calculate the concentration of NAD^+ and NADH present in the solution. Take into account that both compounds absorb at 260 nm, but only NADH absorbs at 340 nm.

Table - Values of molar extinction coefficients for NAD^+ and NADH

	ε (M^{-1} cm^{-1})	
Nucleotide	260 nm	340 nm
NAD^+	18000	≈ 0
NADH	15000	6220

2.4. GENOMIC DNA EXTRACTION AND CHARACTERIZATION

1.- Considering the genomic DNA extraction protocol, answer the following questions: (a) What are the roles of SDS and ethanol in a DNA extraction protocol? (b) How would you check if the extracted DNA is degraded? (c) How can you quantify the amount of DNA obtained? (d) If you ran an agarose gel electrophoresis and the loaded DNA remained in the well (it did not move), what would you change in the gel composition?

2.- Complete the following Table, knowing that the digestion of onion DNA with *BamHI* (an restriction enzyme) was carried out in a total volume of 30 µl and using the relationship that an A_{260} of 1.0 corresponds to 50 µg/ml pure dsDNA.

Component	Stock solution	Required Final Concentration	Required stock Volume (µl)
*Bam*HI	1000 U/ml	0.1 U/µl	
BSA	1 mg/ml	0.1 mg/ml	
Buffer	10X	1X	
DNA	Abs_{260}=1 (dilution 1/10)	1 µg	

Sterile water =
Final volume =

3.- Genomic DNA was extracted from an orange and it was resuspended in 30 µl of sterile water in a 1.5 ml-Eppendorf tube. Then, 2 µl of this solution were diluted with 598 µl of water. The absorbance at 260 nm of the diluted sample was 0.2. (a) Calculate the concentration of the original extracted DNA (b) What volume would you take to digest 30 µg of genomic DNA with

4

EcoRI (a restriction enzyme)? (c) Which amount of genomic DNA would remain in the Eppendorf tube after performing the spectrophotometric measurement and the enzymatic digestion? (d) In order to check if the genomic DNA was properly digested by the restriction enzyme, an agarose gel electrophoresis was carried out. How much agarose is needed to prepare a 0.8% (w/v) agarose gel in a final volume of 50 ml of TAE buffer? (e) What volume do you need of 50X TAE to prepare the gel?

4.- Kiwi DNA was obtained by three different protocols. See in the following Figure the results of the agarose gel electrophoresis analysis of the extracted DNA preparations and discuss about the three DNA extraction protocols. Why is it convenient to run a number of standard DNA markers of known molecular mass on the same agarose gel in which the samples are run? How are these DNA size markers (or DNA ladder) generated?

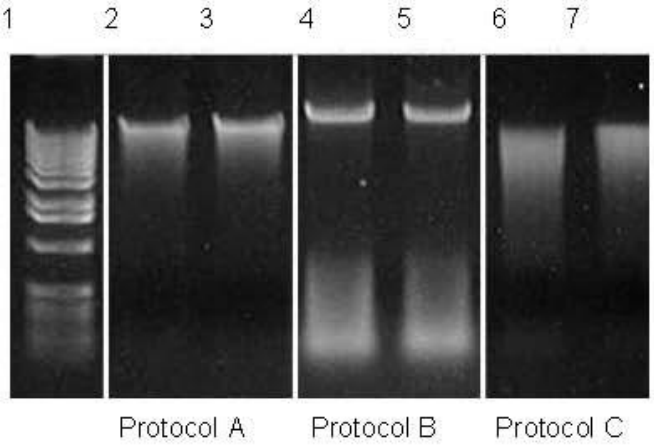


Figure – Photograph showing seven tracks from a 0.8% agarose gel, stained with ethidium bromide and viewed under ultraviolet light. Sample loadings were about 1 µg DNA per track. Track 1, Lambda (λ) DNA cleaved with the enzyme *Bst*EII to generate fragments in the size range from 14.1 kb to 702 pb. Tracks 2 and 3, DNA extracted following protocol A. Tracks 4 and 5, DNA extracted following protocol B. Tracks 6 and 7, DNA extracted following protocol C.

2.5. DETERMINATION OF ENZYME KINETIC PARAMETER

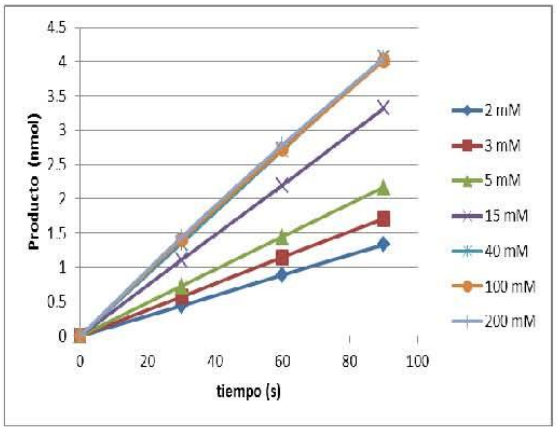
1. - After the purification of an enzyme, the final preparation contained 0.23 g of protein in 5 ml of buffer. Determine the specific activity, expressed as U/mg of protein, knowing that 0.5 ml of the final preparation transformed 4.7 mmol of substrate in 30 s.
2. - A purified hydrolytic enzyme (molecular mass 150 000 Da) was commercially available with the following description: "The enzyme has a Vmax of 3200 U/ mg protein under optimal assay conditions". (a) How many µmoles of substrate can be hydrolyzed in one minute by 1 mg of the purified enzyme? (b) How many nmoles of the enzyme are contained in 1 mg of protein? (c) Assuming that it is pure, determine the molecular activity or turnover number (s^{-1}) for the enzyme.

3.-Test tubes containing different concentrations of substrate were incubated with 1 ug of enzyme (molecular mass 40 000 Da) in a final volume of 4 ml. At intervals of 30 s, a 0.5 ml aliquot was withdrawn from the reaction mixture and the amount of product formed was assessed (see

5

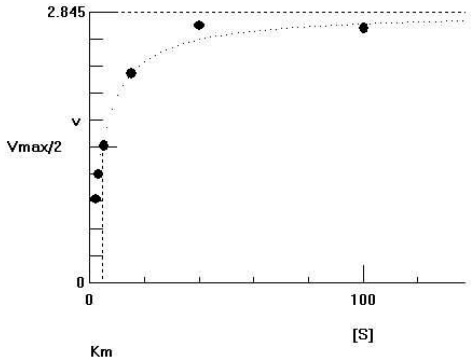
Table). Determine the initial velocity (v_0, µmol product formed per minute) for each substrate concentration.

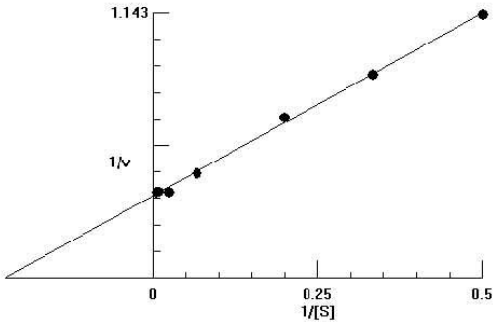
Substrate (mmol/L)	Product (µmol)			
	0 s	30 s	60 s	90 s
2	0	0.44	0.89	1.33
3	0	0.57	1.15	1.71
5	0	0.73	1.45	2.17
15	0	1.11	2.20	3.32
40	0	1.35	2.71	4.06
100	0	1.40	2.73	4.02
200	0	1.44	2.79	4.05



Substrate (mmol/L)	Product (nmol)				
	0 s	30 s	60 s	90 s	
2	0	0.44	0.89	1.33	
3	0	0.57	1.15	1.71	
5	0	0.73	1.45	2.17	
15	0	1.11	2.2	3.32	
40	0	1.35	2.71	4.06	
100	0	1.4	2.73	4.02	
200	0	1.44	2.79	4.05	

4. – Without plotting, analyze the results of the previous question and estimate the values for Vmax and Km. Next, draw a Michaelis-Menten plot and a Lineweaver-Burk plot and determine the values of Vmax and Km.





ANEXO 3B: EJEMPLOS DE CUESTIONES RESUELTAS POR ALUMNOS DEL BLOQUE 2

CUESTIONES.

① Calculate the volume of each stock solution needed to preparate a 10% polyacrylamide - resolving gel (see the Preparation Table for SDS-PAGE gels). How could you prepare a 15-5% polyacrylamide gradient-slab gel?

15% Polyacrylamide ← 3mL Solution A (1/2) ; 1,5mL Solution B (1/4) ; 1,5mL distilled water (1/4) → Separatin gel (6mL)

Separatin gel

10% 3mL/6mL Solution A / 15% = 2mL / 6mL de Solution A.

10% 1,5mL/6mL / 15% = 1mL solution B and 1mL distilled water ; 2mL Solution A

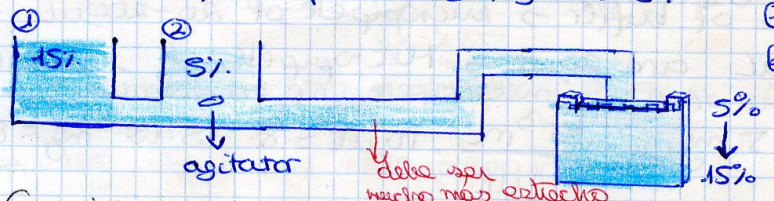
0,35mL Solution A ; 0,625mL Solution C ; 1,5mL distilled water → Concentrating gel (2,56mL)

Concentrating gel.

10% 0,35mL/2,555mL solution A / 15% = 0,23mL / 62,56mL Solution A

0,23mL solution A ; 0,42mL solution C and 1mL distilled water.

To preparate the 15-5% polyacrylamide gradient-slab gel is necesary a system of communicating vessels:



② Consider that there were a 15% - and a 7% - polyacrilamide SDS-PAGE gel in the laboratory. Wich one would you employ to identify a protein of 15KDa? And if the molecular mass of the protein were 150KDa?

To indentify a protein of 15KDa is used the 15% - polyacrilamide SDS-PAGE gel and to identify a protein of 150KDa is used the 7%. Since the larger the percentage will be lower polyacrylamide pore size, so the smaller the mass of the protein must be smaller pore size.

(3) You want to analyze by SDS-PAGE electrophoresis a sample with a protein concentration of 35 mg/mL. For this purpose, an aliquot of the sample is diluted 20-fold with distilled water to a final volume of 500 µL. Then, 30 µL of the 1/20 dilution are mixed with 30 µL of sample buffer (2X), and the mixture is heated for 5 min at 95ºC. Finally, 10 µL and 20 µL of the last preparation are loaded into the gel wells. (a) Describe how you would preparate the required 1/20 dilution, if you had in the laboratory micropipettes for pipetting volumes of 2-20 µL, 20-200 µL and 200-1000 µL. (b) Calculate how many micrograms of protein are loaded into each gel well.

35 mg/mL $\xrightarrow{1/20}$ 500 µL → $\frac{35 \text{ mg/mL}}{20}$ = 1,75 mg/mL in 500 µL

30 µL of 1,75 mg/mL + 30 µL buffer ⇒ 0,875 mg/mL

10 µL of 0,875 mg/mL and 20 µL of 0,875 mg/mL

a) (A) [25 µL of 35 mg/mL → micropipete of 20-200 µL
475 µL of distilled water → micropipete of 200-1000 µL.
30 µL of buffer → micropipete of 20-200 µL.
10 µL and 20 µL → micropipete of 2-20 µL.
30 µL of A → micropipete of 20-200 µL.

b) $0{,}875 \text{ mg/mL} \cdot \frac{10^{-3} \text{ mL}}{1 \text{ µL}} \cdot 10 \text{ µL} = 8{,}75 \cdot 10^{-3} \text{ mg} = 8{,}75 \text{ µg}$

$0{,}875 \text{ mg/mL} \cdot \frac{10^{-3} \text{ mL}}{1 \text{ µL}} \cdot 20 \text{ µL} = 0{,}0175 \text{ mg} = 17{,}5 \text{ µg}$

(4) After an ion-exchange chromatography, a preparation containing 25 mg of protein per milliliter was obtained. To establish the purity of this preparation by SDS-PAGE, you are required to prepare two samples containing 5 µg and 10 µg of protein, respectively. Remember that the maximum loading volume per gel well is 20 microliters and that the sample loading buffer is prepared at double concentration (2x). (a) How much will you dilute the initial preparation with distilled water? (b) Describe the preparation of the two samples that must be loaded in the wells (including sample buffer).

25 mg/mL

* 5 µg/20 µL = 0,25 mg/mL ; $\frac{25\ mg/mL}{0{,}25\ mg/mL} = 100$ (1:100)

$V_1 = \frac{V_2 \cdot C_2}{C_1} = \frac{20\ \mu L \cdot 0{,}25\ mg/mL}{25\ mg/mL} = 0{,}2\ \mu L$ sample

0,2 µL sample + 10 µL Buffer + 9,8 µL distilled water

↓ x25

5 µL sample + 250 µL buffer + 245 µL distilled water

Pipetting 20 µL of this preparation.

* 10 µg/20 µL = 0,5 mg/mL $\frac{25\ mg/mL}{0{,}5\ mg/mL} = 50$ (1:50)

$V_1 = \frac{20\ \mu L \cdot 0{,}5\ mg/mL}{25\ mg/mL} = 0{,}4\ \mu L$ sample.

0,4 µL sample + 10 µL buffer + 6 µL distilled water

↓ x15

6 µL sample + 150 µL buffer + 90 µL distilled water

↓

Pipetting 20 µL of this preparation.

⑤ You are required to load into an SDS-PAGE gel 4 µg of a sample with a protein concentration of 0.8 mg/mL. If the sample loading buffer is three times more concentrated (3x) than the final desired concentration, what volume of protein sample, distilled water and sample loading buffer (3x) should be mixed in order to load a total volume of 15 µL in the well?

4 µg → 0.8 mg/mL V_T = 15 µL.

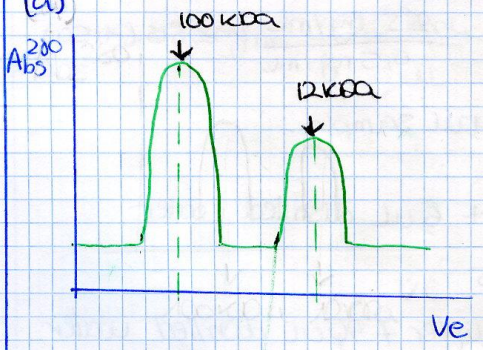
$4\,\mu g \cdot \frac{1\,\mu L}{0.8\,\mu g} = 5\,\mu L$ Muestra (sample)

Volume buffer = 1/3 · 15 µL = 5 µL buffer. ✓

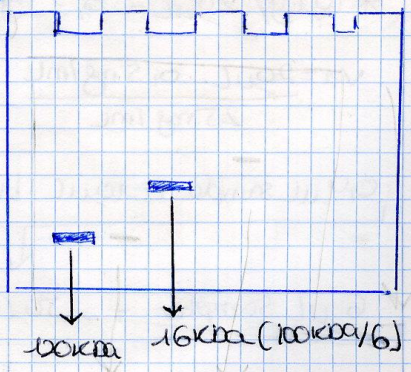
15 µL − 5 µL sample + 5 µL buffer = 5 µL distilled water

⑥ (a) Sketch the expected elution profile (absorbance at 280nm vs elution volume) for a size exclusion ~~molecular~~ chromatography of a protein mixture containing cytochrome C (12kDa) and nucleoside diphosphokinase (100kDa, 6 subunits); identify the peaks in the profile. (b) Sketch the results that would be obtained with the same mixture of proteins if analyze by SDS-PAGE electrophoresis; indicate the migration direction and identify each band.

(a)



(b)



~ Supplementary questions

1.- Considering the genomic DNA extraction protocol, answer the following questions: (a) What are the roles of SDS and ethanol in a DNA extraction protocol? (b) How would you check if the extracted DNA is degraded? (c) How can you quantify the amount of DNA obtained? (d) If you ran an agarose gel electrophoresis and the loaded DNA remained in the well (it did not move), what would you change in the gel composition?

2.- Complete the following Table, knowing that the digestion of onion DNA with *BamHI* (an restriction enzyme) was carried out in a total volume of 30 µl and using the relationship that an A_{260} of 1.0 corresponds to 50 µg/ml pure dsDNA.

Component	Stock solution	Required Final Concentration	Required stock Volume (µl)
*Bam*HI	1000 U/ml	0.1 U/µl	
BSA	1 mg/ml	0.1 mg/ml	
Buffer	10X	1X	
DNA	Abs_{260}=1 (dilution 1/10)	1 µg	

Sterile water = µl
Final volume = µl

3.- Genomic DNA was extracted from an orange and it was resuspended in 30 µl of sterile water in a 1.5 ml-Eppendorf tube. Then, 2 µl of this solution were diluted with 598 µl of water. The absorbance at 260 nm of the diluted sample was 0.2. (a) Calculate the concentration of the original extracted DNA (b) What volume would you take to digest 30 µg of genomic DNA with *EcoRI* (a restriction enzyme)? (c) Which amount of genomic DNA would remain in the Eppendorf tube after performing the spectrophotometric measurement and the enzymatic digestion? (d) In order to check if the genomic DNA was properly digested by the restriction enzyme, an agarose gel electrophoresis was carried out. How much agarose is needed to prepare a 0.8% (w/v) agarose gel in a final volume of 50 ml of TAE buffer? (e) What volume do you need of 50X TAE to prepare the gel?

4.- Kiwi DNA was obtained by three different protocols. See in the following Figure the results of the agarose gel electrophoresis analysis of the extracted DNA preparations and discuss about the three DNA extraction protocols. Why is it convenient to run a number of standard DNA markers of known molecular mass on the same agarose gel in which the samples are run? How are these DNA size markers (or DNA ladder) generated?

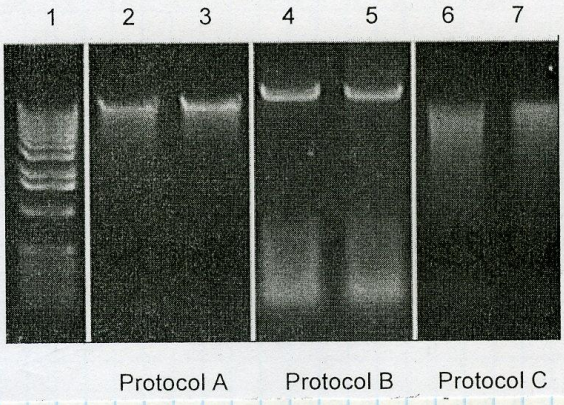


Figure – Photograph showing seven tracks from a 0.8% agarose gel, stained with ethidium bromide and viewed under ultraviolet light. Sample loadings were about 1 µg DNA per track. Track 1, Lambda (λ) DNA cleaved with the enzyme *Bst*EII to generate fragments in the size range from 14.1 kb to 702 pb. Tracks 2 and 3, DNA extracted following protocol A. Tracks 4 and 5, DNA extracted following protocol B. Tracks 6 and 7, DNA extracted following protocol C.

①

a) SDS is a detergent which causes the fragmentation of the plasmatic and inner membranes, the denaturalization of proteins and the liberation of nucleoproteic compounds. To sum up, it acts in favour of the lysis of the cells. Ethanol helps to precipitate the DNA. This is because ethanol (which must be cold) dehydrates the DNA. Moreover, in this process ethanol 'washes' the obtained DNA, releasing it of other kinds of molecules such as protein.

b) In order to check if the extracted DNA is degraded it is possible to run an agarose electrophoresis. If the mentioned DNA is really degraded a smear will appear in the gel when visualising it.

c) To quantify the amount of DNA obtained it is necessary to measure A_{260} in a spectrophotometer, knowing that an $A_{260}=1$ corresponds to 50 µg/mL pure dsDNA.

d) For instance, the agarose concentration could be changed. A lower agarose concentration would mean a bigger pore diameter, so larger DNAs could be analysed.

②

Component	Stock solution	Required final C	Required stock V (µL)
BamHI	1000 U/ mL	0,1 U / µL	3
BSA	1 mg /mL	0,1 mg/mL	3
Buffer	10 X	1X	3
DNA	A_{260} (1/10)	1 µg	2

Sterile water = 19 µL ; V_{final} = 30 µL

- **BamHI**: ~~1000~~ U/mL · 1 mL/10^3 µL = 1 U/µL.

1 U/µL → 0,1 U/µL (V_F = 30 µL) → 0,1 U/µL · 30 µL = 3 U

30 · 1 µL/1 U = **3 µL**

- **BSA**: 1 mg/mL → 0,1 mg/mL (V_F = 30 µL) → 0,1 mg/mL · 30 · 10^{-3} mL = $3 \cdot 10^{-3}$ mg

$3 \cdot 10^{-3}$ mg · 1 mL/mg = **3 µL**

- **Buffer**: 10 X → 1 X (V_F = 30 µL) : 10X/y = 1X/30 µL

y = **3 µL 10X**

- **DNA**: A_{260} = 1,0 → C_{DNA} = 50 µg/mL (1/10)

↓ x10

$C_{DNA\ original}$ = 500 µg/µL

500 µg/µL → 1 µg (in ~~30~~ µL)

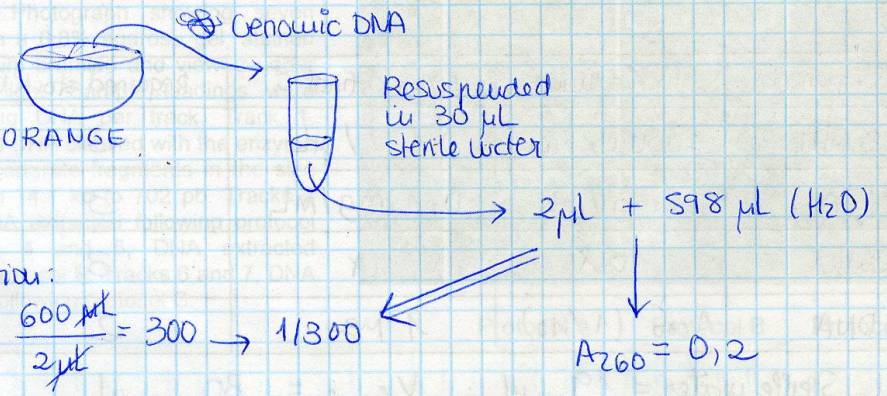
1 µg · 1 µL/500 µg =

= **2 µL DNA**

- H_2O (sterile water) ⇒ $V_{H_2O} = V_{final} - V_{BamHI} - V_{BSA} - V_{Buffer} - V_{DNA}$

V_{H_2O} = 30 µL − (3+3+3+2) µL = 19 µL

③



Dilution:

F = 600 µL/2 µL = 300 → 1/300

a) $A_{260} = 1{,}0 \leftrightarrow C_{DNA} = 50\ \mu g/mL$:

$\frac{1{,}0}{50\ \mu g/\mu L} = \frac{0{,}2}{x}$; $x = C_{DNA} = 10\ \mu g/mL$ (diluted: 1/300)

$\times 300$ ↓

$C_{DNA\ original} = 3.000\ \mu g/mL$

b) $30\ \mu g \cdot \frac{1\ mL}{3000\ \mu g} = 0{,}01\ mL = 10\ \mu L$ to digest 30 µg DNA

c) $V_T = 30\ \mu L$

$V_{A260} = 2\ \mu L$

$V_{digestion} = 10\ \mu L$

$V_{left} = 30\ \mu L - 2\ \mu L - 10\ \mu L =$ 18 µL DNA

$18\ \mu L \cdot 10^{-3}\ \frac{mL}{\mu L} \cdot 3.000\ \mu g/\mu L =$ 54 µg DNA

d) 0,8 % (w/v) agarose gel: $V_T = 50\ mL$.

$\frac{0{,}8\ g}{100\ mL} \cdot 50\ mL =$ 0,4 g of agarose

e) $V_T = 50\ mL \rightarrow 50/50 =$ 1 mL of TAE buffer

④ It is convenient to run a number of standard DNA markers of known molecular mass on the same agarose gel in which the samples are run because these markers might run differently in different gels. For instance, the agarose concentration might not be exactly the same; the value of mA/gel may change in different gels... So, it is better to run the DNA markers in each gel because as their migration distance might change it is directly related to the samples being run.

DNA size markes (or DNA ladder) are obtained by digestion of know DNAs, such as λ (lambda) phage or φ29 phage, with some kind of restriction enzyme (Hind III, BstE II...)

Discussion of the gel:

Protocol A: DNA samples (2 and 3) appear like defined bands on top of the gel. Also, it is possible to see under these two bands a light smear, but compared to the ones observed in protocols B and C these ones are less important. In addition, in the lower part of the gel there isn't any kind of band or smear.

Protocol B: two defined bands appear on top of the gel, with a really good resolution. Nevertheless, the are big smears in the lower part of the gel (large molecular weights), which may be caused by the presence of RNA. So, using protocol B it looks like RNA contamination is observed, maybe because RNA is not completely removed from the DNA sample.

Protocol C: DNAs isolated by this protocol are degraded, since there is not a well-defined band but a big smear in the middle of the gel. Some kind of DNase must have acted on the DNA samples. If protocol C includes some endonuclease, the results shown are really good; this would mean that the DNA are high quality ones. By contrast, if protocol C did not include itself an endonuclease, these DNAs would not be good ones, once the have been digested by an endonuclease that doesn't belong to protocol C.

SUPPLEMENTARY QUESTIONS

1. - After the purification of an enzyme, the final preparation contained 0.23 g of protein in 5 ml of buffer. Determine the specific activity, expressed as U/mg of protein, knowing that 0.5 ml of the final preparation transformed 4.7 mmol of substrate in 30 s.

2. - A purified hydrolytic enzyme (molecular mass 150 000 Da) was commercially available with the following description: "The enzyme has a Vmax of 3200 U/ mg protein under optimal assay conditions". (a) How many µmoles of substrate can be hydrolyzed in one minute by 1 mg of the purified enzyme? (b) How many nmoles of the enzyme are contained in 1 mg of protein? (c) Assuming that it is pure, determine the molecular activity or turnover number (s^{-1}) for the enzyme,

3.-Test tubes containing different concentrations of substrate were incubated with 1 ug of enzyme (molecular mass 40 000 Da) in a final volume of 4 ml. At intervals of 30 s, a 0.5 ml aliquot was withdrawn from the reaction mixture and the amount of product formed was assessed (see Table). Determine the initial velocity (v_0, µmol product formed per minute) for each substrate concentration.

Substrate (mmol/L)	Product (µmol)			
	0 s	30 s	60 s	90 s
2	0	0.44	0.89	1.33
3	0	0.57	1.15	1.71
5	0	0.73	1.45	2.17
15	0	1.11	2.20	3.32
40	0	1.35	2.71	4.06
100	0	1.40	2.73	4.02
200	0	1.44	2.79	4.05

4. – Without plotting, analyze the results of the previous question and estimate the values for Vmax and Km. After this, draw a Michaelis-Menten plot and a Lineweaver-Burk plot and determine the values of Vmax and Km.

1. 0.5 ml ⟶ 4.7 mmol S / 30s

= 4700 µmol S / 30 s

$4700 \frac{\mu mol\ S}{30s} \cdot \frac{60s}{1min} = 9400\ \mu mol\ S/min$

0.23 g of protein ⟶ 5 ml of buffer.

0.5 ml ⟶ 9400 µmol S/min

5 ml ⟶ x

$x = \frac{9400\ \mu mol\ S/min \cdot 5\ ml}{0.5\ ml} = 94000\ \mu mol\ S/min$

(94000 U)

0.23 g of protein ⇒ 230 mg of protein

$$\text{Specif. Activity} = \frac{94000\ U}{230\ mg} = 408.69\ U/mg \simeq 408.7\ U/mg$$

2. M_r = 150 000 Da ⟶ PURIFIED enzyme

v_{max} = 3200 U/mg of protein

(a) 3200 μmoles of substrate (conclusion drawn from the v_{max} definition).

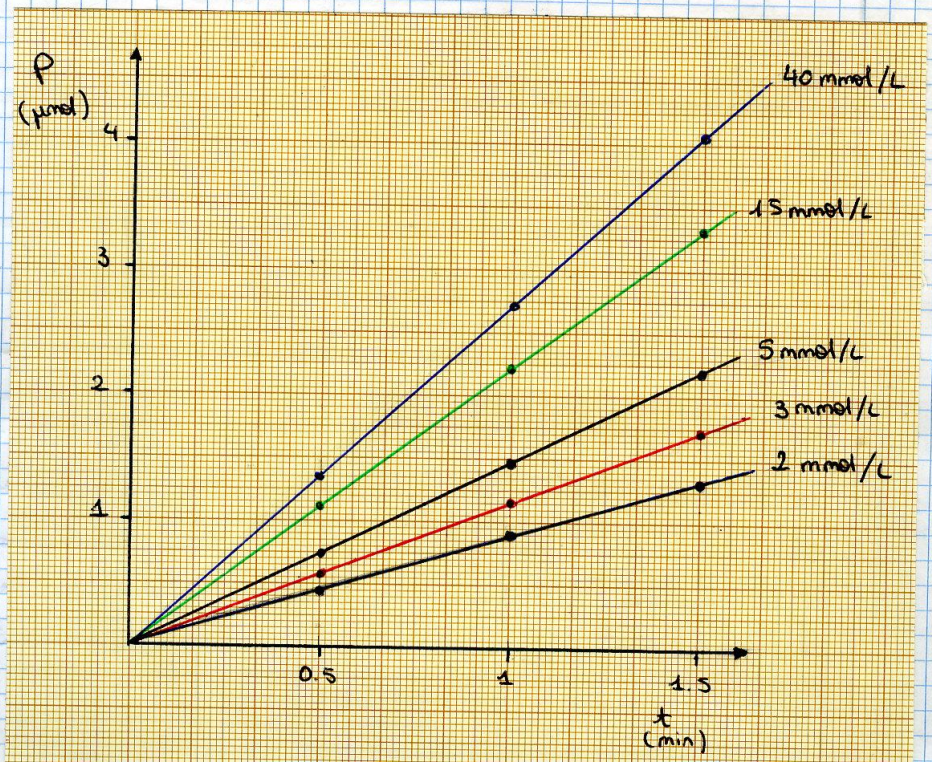
(b) 1 mg of protein = 1 mg of enzyme (given that it has been previously purified).

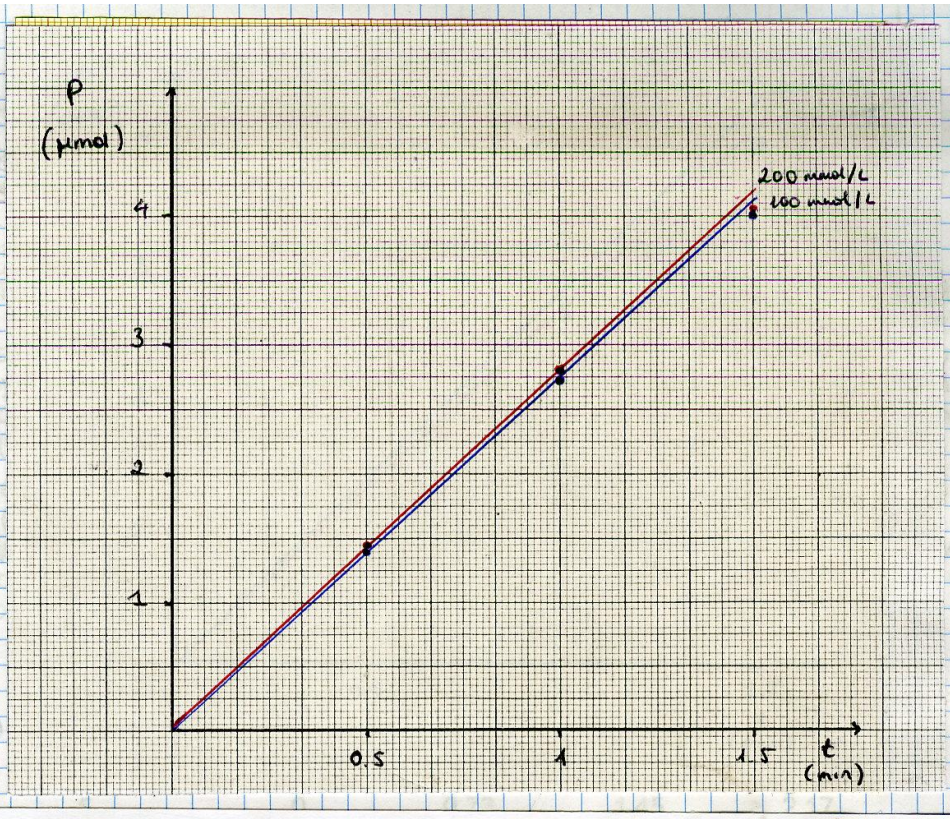
$$\frac{1 \cdot 10^{-3}\ g \text{ of protein}}{150\ 000\ g/mol} = 6.666 \cdot 10^{-9}\ mol = 6.67\ nmoles$$

6.67 nmoles of enzyme / 1 mg of protein

(c) $$T.N. = \frac{3200\ \mu mol\ S/min \cdot mg}{6.67 \cdot 10^{-3}\ \mu mol\ E/mg} \cdot \frac{1\ min}{60\ s} = 7996\ s^{-1}$$

3.





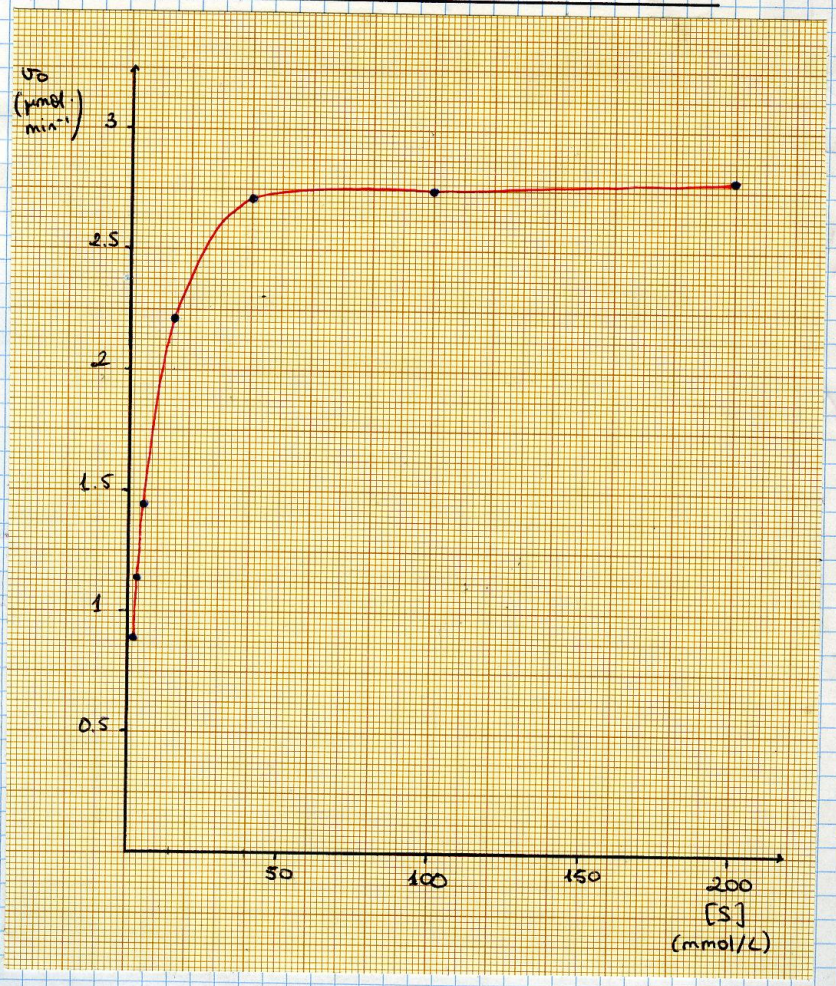
Graph 9. Variation of product concentration with time for [substrate] = 100 mmol/L and [substrate] = 200 mmol/L.

Substrate Concentration (mmol/L)	Regression (least squares adjustment)	v_0 (µmol / min)
2	$y = 0.888x - 0.001$	0.888
3	$y = 1.142x + 0.001$	1.142
5	$y = 1.446x + 0.003$	1.446
15	$y = 2.210x$	2.210
40	$y = 2.708x - 0.001$	2.710

* For [substrate] = 100 mmol/L and [substrate] = 200 mmol/L I only used three points to make the least squares adjustment: the points corresponding to t = 1.5 min diverted from linearity.

Substrate Concentration (mmol/L)	Regression (Least squares adjustment)	v_0 (μmol/min)
100	$y = 2.730 + 0.012$	2.73
200	$y = 2.79 + 0.015$	2.79

4. Although the leap between a substrate concentration of 100 mmol/L and another of 200 mmol/L is considerable, their respective v_0 don't differ that much from each other. That probably indicates that the v_{max} is around 2.79 μmol/min. If it were the case, the K_M would be between 3 and 5 mmol/L (given that the K_M corresponds to the substrate concentration at which the velocity is a half from v_{max}). Closer to 5 than to 3 mmol/L, given that $\frac{1}{2} v_{max} = 1.395$ and that the v_0 at [substrate] = 5 is equal to 1.446 μmol/min.



Graph 10. Michaelis-Menten plot.

Para la proteína problema y la hemoglobina, se sabe que:

Proteína problema { - Ve = 42.5 ml
- Kav = 0'49

Hemoglobina { - Ve = 36.5 ml
- Kav = 0'33

Sustituyendo en la ecuación de la recta estos valores de Kav se obtiene que:

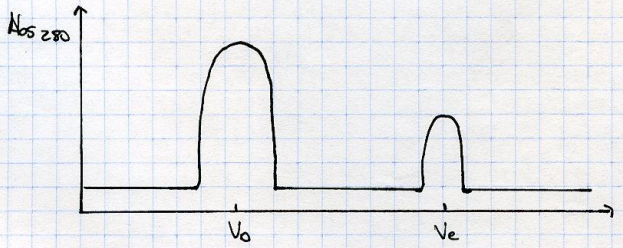
Proteína problema ⇒ Masa mol = 12.3 KDa
Hemoglobina ⇒ Masa mol. = 22.4 KDa

Supplementary questions

① A protein (M = 240000 Da) dissolved in 1M potassium phosfate buffer, pH 7.5, was loaded on a Sephadex G-25 column, previously equilibrated with 50 mM potassium phosfate buffer pH 7.5. This equilibration buffer was also used for the elution step. In which volume will you expect to elute the protein? What will be the molarity of the phosphate buffer in which the protein elutes?

Due to the high mass molecular of the protein, this would be included of the gel, with a Kav = 0, and therefore the elution volume would be the same as the exclusion one.
The molarity of the phosphate buffer in which the protein elutes will be, equally, 10 mM

② A mixture containing three proteins was loaded on a Sephadex G-150 column (fractionation range for globular proteins 5000 - 300000 Da). The figure shows the chromatographic elution profile. Why are there only two peaks in the elution profile? What techniques could you use to determine the protein composition of each peak? How could you try to separate the three proteins?



There are only three peaks in the elution profile because one of the proteins of the mixture has a molecular mass which is superior than 300 KDa or inferior than 5 KDa, and therefore it elutes in the exclusion or total volume, so

it doesn't appear as a peak in the elution profile.
To determine the protein composition of each peak we could use mass spectrometry technique, and we could try to separate the three proteins by using another gel with a smaller fractionation range.

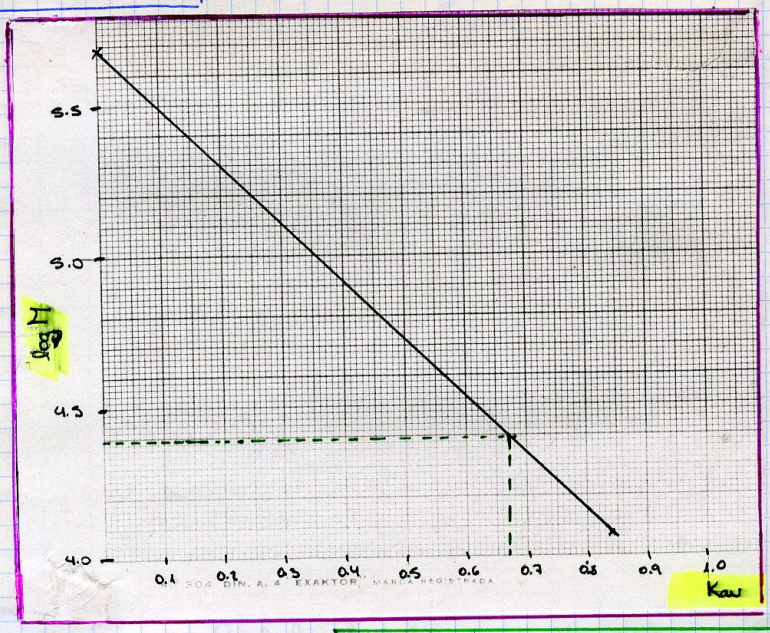
③ The molecular mass of an enzyme isolated from Acinetobacter sp. was determined by size exclusion cromatography. The following Kav values were obtained for standard proteins of known molecular mass. Estimate the molecular mass of the bacterial enzyme, knowing that its Kav value is 0.67

We represent log M vs Kav of each standard protein using the datum of the given table.

Protein	Molecular mass (Da)	Log M	Kav
Cytochrome C	12000	4.08	0.840
Lysozyme	14000	4.15	0.790
Ovalbumin	43000	4.65	0.550
Bovine serum albumin	66000	4.82	0.440
Alcohol dehydrogenase	160000	5.20	0.260
Pyruvate Kinase	235000	5.37	0.156

By using the method of least-squares, we obtain the following equation:

$Y = 5'68 - 1'92x$



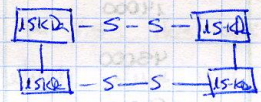
Replacing Kav = 0.67, we obtain molecular mass of the protein = 24547 Da

G5

④ [Determination of the number of subunits]. A protein was purified to homogeneity. By size exclusion cromatography its molecular mass was estimated to be 60 KDa in the absence of urea, and 30 KDa when the chromatography was performed in the same buffer containing 6 M urea. When the chromatography was repeated in the presence of 6M urea and 10mM β-mercaptoethanol, a single peak corresponding to 15 KDa was detected. Describe the molecular structure of the protein.

By doing just the size exclusion chromatography, we obtain a molecular mass of 60KDa. Then, if we use 6M urea, we obtain a 30KDa molecular mass. This one corresponds to the monomer and 60KDa corresponds to the total protein, which is supposed to be a dymer.

Then, by using β-mercaptoethanol, we obtain a peak of 15KDa. This shows us that the protein has 4 subunits, two of them united by an -S-S-.



SUPPLEMENTARY QUESTIONS

1- Calculate the volume of each stock solution needed to prepare a 10% polyacrylamide-resolving gel (see the Preparation Table for SDS-PAGE gels). How could you prepare a 5-5% polyacrylamide gradient-slab gel?

To prepare 10% polyacrylamide resolving gel we need:
3.0mL of A solution
1.5mL of B solution
1.5mL of H_2O
30µL of Persulfate 10%(p/v)
75µL of TEMED 10%(p/v)

With this, we know that we need for a 10% polyacrylamide resolving gel.
2.0mL of A solution
1.0mL of B solution
1.0mL of H_2O
20µL of Persulfate 10% (p/v)
50µL of TEMED 10% (p/v)

2- Consider that there were a 15%- and a 7%-polyacrylamide SDS-PAGE gel in the laboratory, which one would you employ to identify a protein of 15 kDa? And if the molecular mass of the protein were 150 kDa?

To identify a protein of 15kDa I would employ a 15% polyacrylamide gel because when we want to separate small proteins we have to use a polyacrylamide gel whose percentage was greater. If the percentage is greater the pores will be smaller because the concentration will be bigger. To identify a protein of 150 kDa I would use a 7% polyacrylamide gel because with a smaller percentage, the gel will have less concentration and the pores will be bigger and they will let pass a big protein.

3- You want to analyze by SDS-PAGE electrophoresis a sample with a protein concentration of 35 mg/ml. For this purpose, an aliquot of the sample is diluted 20-fold with distilled water to a final volume of 500 µl. Then, 30 µl of the 1/20 dilution are mixed with 30 µl of sample buffer (2X), and the mixture is heated for 5 min at 90 °C. Finally, 10 µl and 20 µl of the last preparation are loaded into the gel wells. (a) Describe how you would prepare the required 1/20 dilution, if you had in the laboratory micropipettes for pipetting volumes of 2-20 µl, 20-200 µl and 200-1000 µl. (b) Calculate how many micrograms of protein are loaded into each gel well.

a) 20 = 500 µL V = 25 µL

We put 25 µL of the protein and 475 µl of destilled water.

We put the 25µL with the (2 0-200 µL) micropipette and the 475µL with the 200-1000µL micropipette

b) C 1/20 → $C_1 V_1 = C_2 V_2$

35 mg/mL · 25 = C_2 · 500

C_2 = 1'75 mg/mL

With buffer

$C_1 \cdot V_1 = C_2 \cdot V_2$

1'75 mg/mL · 30 = C_2 · 60

C_2 = 0'875 mg/mL

We put 10 µL → 10^{-3} mL We put 20 µL → $20 \cdot 10^{-3}$ mL

4- After an ion-exchange chromatography, a preparation containing 25 mg of protein per milliliter was obtained. To establish the purity of this preparation by SDS-PAGE, you are required to prepare two samples containing 5 µg and 10 µg of protein, respectively. Remember that the maximum loading volume per gel well is 20 microliters and that the sample loading buffer is prepared at double concentration (2X). (a) How much will you dilute the initial preparation with distilled water? (b) Describe the preparation of the two samples that must be loaded in the wells (including sample buffer).

25 mg/mL

Sample 1

$C_1 = \frac{25\ mg}{mL} \cdot \frac{1\ mL}{1000\ \mu L} \cdot \frac{1000\ \mu g}{1\ mg} = 25\ \mu g/\mu L$

$C_2 = \frac{5\ \mu g}{20\ \mu L} = 0'25\ \mu g/\mu L$

$C_1 \cdot V_1 = C_2 \cdot V_2$

$V_1 = \frac{0'25\ \mu g/\mu L \cdot 20\ \mu L}{25\ \mu g/\mu L} = 0'2\ \mu L$ of the initial preparation

$F = \frac{20\ \mu L}{0'2\ \mu L} = 100$

We have to do a 1/100 dilution of the initial preparation

0'2 µL initial preparation — 9'8 µL of water (x2) = 19'6 µL

5 µL — 245 µL

10 µL + 10 µL of sample buffer 2x

250 µL + 250 µL

We have to add 0'2 µL of initial preparation, 9'8 µL of water and 10 µL of sample buffer 2x in the well (x2).

Sample 2

$C_2 = \frac{10\ \mu g}{20\ \mu L} = 0'5\ \mu g/\mu L$

$C_1 \cdot V_1 = C_2 \cdot V_2$

$V_1 = \frac{0'5\ \mu g/\mu L \cdot 20\ \mu L}{25\ \mu g/\mu L} = 0'4\ \mu L$

$F = \frac{20\ \mu L}{0'4\ \mu L} = 50$

We have to do a 1/25 dilution of the initial preparation

We have to add in the well 0'4 µL (x20 = 8 µL) of the initial preparation, 9'6 µL (192 µL) of destilled water and 10 µL (x20 = 200 µL) of the sample buffer).

5- You are required to load into an SDS-PAGE gel 4 µg of a sample with a protein concentration of 0.8 mg/ml. If the sample loading buffer is three times more concentrated (3X) than the final desired concentration, what volume of protein sample, distilled water and sample loading buffer (3X) should be mixed in order to load a total volume of 15 µl in the well?

15 µl in total
5 µl with the buffer and 10 µl with the preparation

$C_2 = \frac{4\ \mu g}{15\ \mu L} = 0'27\ \mu g/\mu l$

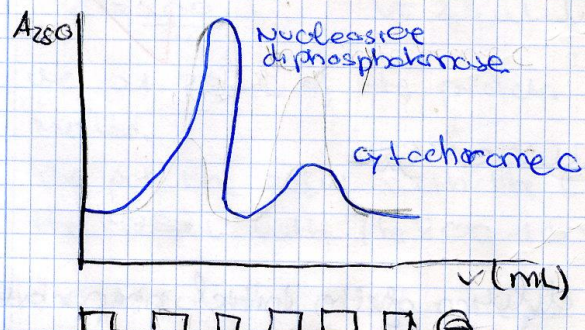
0'8 mg/ml = 0'8 µg/µl

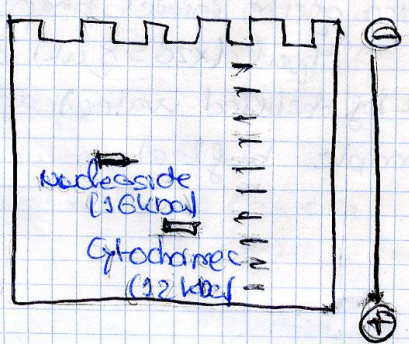
$C_1 \cdot V_1 = C_2 \cdot V_2$

$V_1 = \frac{C_2 \cdot V_2}{C_1} = \frac{0'27\ \mu g/\mu l \cdot 15\ \mu l}{0'8\ \mu g/\mu l} = 5'06 = 5\ \mu l$

We have to add 5 µl of the initial preparation and 5 µl of destilled water and 5 µl of buffer in the well.

6- (a) Sketch the expected elution profile (absorbance at 280 nm *vs* elution volume) for a size exclusion chromatography of a protein mixture containing cytochrome c (12 kDa) and nucleoside diphosphokinase (100 kDa, 6 subunits); identify the peaks in the profile. (b) Sketch the results that would be obtained with the same mixture of proteins if analyzed by SDS-PAGE electrophoresis; indicate the migration direction and identify each band.





100/6 = 16'67 kDa
SDC → Desnaturalizar

ANEXO 4: GLOSARIO

Scientific vocabulary

Laboratory of Biochemistry and Molecular Biology I

2014-2015

Degree in Biochemistry, UCM

MATERIAL BASICO DE LABORATORIO / BASIC LABORATORY EQUIPMENT

Material de vidrio,	**Glassware**
bureta,	buret(te)
embudo,	(conical) funnel
erlenmeyer,	erlenmeyer flask
matraz,	flask
matraz aforado,	volumetric flask
pipeta,	pipet(te)
probeta,	graduated cylinder
tubo de ensayo,	test tube
vaso de precipitados,	beaker

Dispositivos,	**Devices**
aspirapipetas,	pipette filling device
cuentagotas,	dropper
escobilla,	brush
espátula,	spatula
frasco lavador,	wash bottle
gradilla,	rack, holder
mechero Bunsen,	bunsen burner
papel de filtro,	filter paper
pinzas,	forceps, tweezers
rotulador de vidrio,	glass marker
tapón,	stopper, cap
tubo de ensayo,	test tube

Instrumentos,	**Instruments**
agitador,	shaker
agitador magnético,	magnetic stirrer
agita-tubos,	Vortex
balanza,	balance
baño de agua termostatizado,	thermostated water bath
bomba de vacío,	vacuum pump
centrífuga,	centrifuge
congelador,	freezer
estufa,	oven
microscopio,	microscope
nevera,	refrigerator, fridge
placa calefactora,	hot plate
pH-metro,	pHmeter
temporizador,	timer
termómetro,	thermometer

CHAPTER 1.1 BIOCHEMISTRY LABORATORY

Seguridad,	**Safety**
bata de laboratorio,	lab coat
contenedor de residuos,	disposal container
extintor,	fire extinguisher
gafas de seguridad,	safety googles
guantes,	gloves
máscara,	face mask
peligro,	danger
riesgo biológico,	biohazard
vertido,	spillage

Estadística,	**Statistic**
coeficiente de variación,	coefficient of variation (CV)
desviación estándar,	standard deviation (SD)
exactitud,	accuracy
media,	mean
precisión,	precision

Vocabulario,	**Vocabulary**
cantidad,	amount
cuaderno de laboratorio,	laboratory notebook
densidad,	density
masa,	mass
micropipeta,	micropipette
peso,	weight
pipeta de vidrio,	graduated pipette
puntas (de micropipeta),	tips
volumen,	volume

Verbos,	**Verbs**
añadir,	to add
dibujar,	to plot
medir,	to measure
pesar,	to weigh
pipetear,	to pipette
tarar,	to tare

CHAPTER 1.2 SOLUTIONS AND pH

Unidades,	**Units**
gramo,	gram (g)
litro,	liter (l)
metro,	meter (m)
minuto,	minute (min)
mol,	mole (mol)
molaridad,	molarity (M)
segundo,	second (s)

Vocabulario,	**Vocabulary**
agua desionizada,	deionized water
agua destilada,	distilled water
concentración,	concentration
curva de titulación,	titration curve
disolución,	solution
disolución de calibrado,	standard solution
disolución de trabajo,	working solution
disolución madre,	stock solution
disolvente,	solvent
electrodo,	electrode
gota a gota,	dropwise
ionización,	ionization
indicador de pH,	pH indicator
mezcla,	mixture
producto químico,	chemical
propiedades ácido-base,	acid-base properties
reactivo,	reagent
soluto,	solute
tampón,	buffer

Verbos,	**Verbs**
agitar,	to stir
almacenar,	to store
conservar,	to keep, kept, kept
disolver,	to dissolve
lavar un recipiente,	to wash out
llenar,	to fill
mezclar,	to mix
transferir,	to transfer

CHAPTER 1.3 DILUTIONS AND STANDARD CALIBRATION CURVES

Resultados,	**Results**
cálculo,	calculation
cifra significativa,	significant figure
eje,	axis
error,	error
escala,	scale
figura,	figure, graph
mínimos cuadrados,	least-squares
número,	number, figure
observación,	data point
pendiente,	slope
porcentaje,	percentage
punto de corte,	intercept
símbolo,	symbol
tabla,	table

Vocabulario,	**Vocabulary**
alícuota,	aliquot
blanco,	blank
control,	control
curva de calibrado,	standard curve
dilución,	dilution
diluciones seriadas,	serial dilutions
factor de dilución,	dilution factor (DF)
intervalo,	range
límite de detección,	detection limit
muestra,	sample
patrón,	standard
referencia,	reference
relación lineal,	linear relationship
réplicas,	replicates
sensibilidad,	sensitivity

Verbos,	**Verbs**
ajustar,	to adjust
calcular,	to calculate
calibrar,	to calibrate
comprobar,	to check, to verify
detectar,	to detect
diluir,	to dilute
ensayar, analizar	to assay, to analyze
medir,	to measure
interpolar,	to interpolate
rotular,	to label

CHAPTER 1.4 QUANTITATIVE DETERMINATION OF PROTEIN CONCENTRATION

Vocabulario,	Vocabulary
absorbancia,	absorbance
coeficiente de extinción molar,	molar extinction coefficient
colorante,	dye
colorimetría,	colorimetry
colorímetro,	colorimeter
cubeta,	cuvette, cell
ensayo colorimétrico,	colorimetric assay
ensayo espectrofotométrico,	spectrophotometric assay
espectrofotómetro,	spectrophotometer
fundamento,	principle
interferencias,	interferences
luz monocromática,	monochromatic light
longitud de onda,	wavelength
máximo,	maximum (a)
método,	method
paso óptico,	light path
proteína,	protein
transmitancia,	transmittance
ultravioleta,	ultraviolet (UV)
visible,	visible (VIS)

CHAPTER 1.5 BIOLOGICAL SAMPLE PREPARATION

Vocabulario,	Vocabulary
agitación,	stirring, shaken
congelación-descongelación,	freeze-thawing
desalado,	desalting
dialisis,	dialysis
disolventes orgánicos,	organic solvents
formación de espuma,	foaming
fraccionamiento,	fractionation
fuerza iónica,	ionic strength
liofilización,	lyophilization, freeze-drying
manejo,	handling
membrana semipermeable,	semipermeable membrane
muestra,	sample
precipitación,	precipitation
sedimento,	sediment
sobrenadante,	supernatant
solubilidad,	solubility
sulfato amónico,	ammonium sulfate
ultrafiltración,	ultrafiltration
técnica,	technique
temperature ambiente,	room temperature
tubo de centrífuga,	centrifuge tube

Verbos,	Verbs
calentar,	to heat
centrifugar,	to centrifuge,
concentrar,	to concentrate
congelar,	to freeze, froze, frozen
descongelar,	to thaw
desechar	to get rid of, to dispose of
eliminar	to remove
enfriar,	to cool
evitar,	to avoid
incubar,	to incubate
liofilizar,	to freeze-dry
llenar,	to fill
precipitar,	to precipitate
resuspender,	to suspend
separar,	to separate
tratar,	to treat

CHAPTER 2.1 MOLECULAR EXCLUSION CHROMATOGRAPHY

Vocabulario,	**Vocabulary**
aguja,	needle
burbujas de aire,	air bubbles
columna,	column
cromatograma,	chromatogram
decantación,	decantation
eluyente,	effluent, eluant
exclusión molecular,	molecular exclusion
fase móvil,	mobile phase
fracción,	fraction
grietas,	cracks
intervalo de fraccionamiento,	fractionation range
jeringa,	syringe
lana de vidrio,	glass wool
partículas finas,	fines
pasta,	slurry
patrones de masa molecular,	molecular mass standards
pinza (abrazadera)	clamp holder
pinza Hoffman,	Hoffman clamp
poro,	pore
reservorio,	reservoir
soporte (columna)	stand
suspensión,	suspension
tamaño de partícula,	particle size
tubo de entrada,	inlet tubing
tubo de salida,	outlet tubing
varilla de vidrio,	glass rod
velocidad de flujo,	flow rate
volumen de elución,	elution volume (Ve)
volumen de exclusion,	void volume (Vo)
volumen de lecho,	bed volume
volumen total,	total volume (Vt)

Verbos,	**Verbs**
aplicar, cargar,	to apply, to load
calibrar,	to calibrate
cromatografiar,	to chromatograph
equilibrar,	to equilibrate
estimar,	to estimate
eluir,	to elute
empaquetar,	to pack
inyectar,	to inject
montar (sistema)	to assemble
recoger,	to collect

CHAPTER 2.2 SDS-PAGE ELECTROPHORESIS

Vocabulario,	**Vocabulary**
acrilamida,	acrylamide
amperaje,	current, amperage
banda,	band
carga,	charge
desnaturalizante,	denaturing
detergente,	detergent
discontinuo,	discontinuous
electroforesis,	electrophoresis
entrecruzamiento,	cross-linking
espaciador,	spacer
fijación,	fixing
frente,	front
gel en placa,	slab gel
gel concentrante,	stacking gel
gel separador,	resolving gel
marcador de frente,	tracking dye
matriz,	matrix
migración,	migration
monómero,	monomer
movilidad,	mobility
peine,	comb
placa de vidrio,	glass plate
pocillo,	well
polímero,	polymer
polo,	pole
proteínas patrones,	protein markers
relación carga/masa,	charge to mass ratio
resolución,	resolution
tinción,	staining
voltaje,	voltage

Verbos,	**Verbs**
correr (gel),	to run
desteñir,	to destain
lavar,	to wash
migrar,	to migrate
preparar (gel)	to cast
polimerizar,	to polymerize
remojar,	to soak
teñir,	to stain
unirse,	to bind

CHAPTER 2.3 UV-VIS SPECTROSCOPY

Vocabulario,	Vocabulary
ajuste de cero,	zero adjustment
compartimento de muestras,	sample chamber
cromóforo,	chromophore
cubeta desechable,	disposable cuvette
cubeta de cuarzo,	quarzt cuvette
cubeta de cristal,	glass cuvette
detector,	detector
dispersion,	scattering
ecuación de Lambert-Beer,	Beer-Lambert Law
espectro de absorción,	absortion spectrum (a)
filtro,	filter
fuente de luz,	light source
lámpara,	lamp
lente,	lens
línea base,	base line
pico,	peak
rayo emergente,	emergent beam
rayo incidente,	incident beam
referencia,	blank, reference
registro,	recorder
soporte de cubetas,	cuvette/cell holder
tapa,	lid
turbidez,	turbidity

Verbos,	Verbs
apagar,	to switch off
calentar (aparato)	to warm
colocar,	to place
encender	to switch on
manejar,	to handle
mostrar,	to display
obedecer,	to obey

CHAPTER 2.4 DNA ISOLATION AND CHARACTERIZATION

Vocabulario,	Vocabulary
agarosa,	agarose
aislamiento,	isolation
capa,	layer
disociación,	dissociation
doble hebra,	double strand (ss)
desenrollamiento,	unwinding
enrollamiento,	spooling
estrés mecanico,	mechanical stress
extracción,	extraction
fase acuosa,	aqueous phase
fase orgánica,	organic phase
frágil,	fragile
fragmento,	fragment
iones metálicos,	metal ions
interfase,	interface
lisis,	lysis
perturbación,	disruption
pureza,	purity
superficie,	surface
temperarura de fusion,	melting temperature (Tm)

Verbos,	Verbs
caracterizar,	to characterize
derretir, fundir,	to melt
desbaratar,	to disrupt
enrollar,	to spool
partir, dividir,	to cleave
visualizar,	to visualize

CHAPTER 2.5 ENZYME KINETIC PARAMETERS

Vocabulario,	Vocabulary
análisis cinético,	kinetic analysis
asíntota,	asymptote
catálisis,	catalysis
cinetica no lineal,	non-linear kinetics
complejo	complex
constante de Michaelis,	Michaelis constant
ecuación	equation
hiperbólico,	hyperbolic
número de recambio,	turnover number
parámetros,	parameters
producto,	product
reacción,	reaction
selectividad,	selectivity
sitio active,	active site
sustrato,	substrate
tangente,	tangent
velocidad inicial,	initial rate
velocidad máxima,	maximal velocity

Verbos,	Verbs
catalizar,	to catalyze
convertir,	to convert
derivar,	to derive, to come from
estimar,	to estimate
evaluar,	to evaluate, to assess
examinar,	to examine, to study
investigar,	to investigate
resultar,	to result
saturar,	to sature

SECTION III ENZYME PURIFICATION

Vocabulario,	Vocabulary
actividad enzimática,	enzyme activity
actividad específica,	specific activity
actividad total,	total activity
carga neta,	net charge
clara de huevo,	egg white
criterios de pureza,	criteria for purity
cromatografía de intercambio iónico,	ion exchange chromatography
curso temporal,	time course
diagrama de flujo,	flow chart
dispersion (luz),	scattering
ensayo cinético,	kinetic assay
ensayo enzimático,	enzyme assay
estrategía,	strategy
etapa de purificación,	separation step/stage
extracto crudo,	crude extract
factor de purificación,	purification factor
fuente biológica,	biological source
gasa,	gauze
homogeneizado,	homogenate
huevo de gallina,	hens´egg
impurezas,	impurities
intercambiador de cationes,	cation exchanger
pared celular,	cell wall
purificación relativa,	fold purification
rendimiento (global),	(overall) yield
resina,	resin
restos, desechos,	debris
solubilidad,	solubility
tabla de purificación,	purification table
yema,	yolk

Verbos,	Verbs
controlar,	to control
convertir,	to transform, to convert into
detectar,	to detect
desarrollar,	to develop
filtrar,	to filter
fraccionar,	to fractionate
juntar, combin ar,	to pool (fractions)
homogeneizar,	to homogenize
mezclar,	to mix
purificar,	to purify

SECTION IV KINETIC CHARACTERIZATION OF ENZYMES

Vocabulario,	**Vocabulary**
ajuste,	adjustment
análogo del sustrato,	substrate analogue
condiciones ambientales,	environmental conditions
constante de disociación,	dissociation constant
constante de inhibición,	inhibition constant (Ki)
constante de velocidad,	rate constant
control,	control
curva de progreso,	progress curve
dependencia con,	dependence on
dependiente del tiempo,	time-dependent
diseño experimental,	experimental design
efectos de temperatura,	temperature effects
elección,	choice
ensayo acoplado,	coupled assay
ensayo (dis)continuo,	(dis)continuous assay
ensayo de tiempo fijo,	fixed-time assay
ensayo continuo,	continuous assay
estabilidad,	stability
estado estacionario,	steady state
estado de transición,	transition state
estequiometría,	stoichiometry
inactivación,	inactivation
inhibición acompetitiva,	uncompetitive inhibition
inhibición competitiva,	competitive inhibition
inhibición mixta,	mixed inhibition
inhibición por producto,	product inhibition
inhibición (ir)reversible,	(ir)reversible inhibition
inhibidor,	inhibitor
intervalo,	range
inverso,	reciprocal
mecanismo catalítico,	catalytic mechanism
orden obligatorio,	compulsory order
pH óptimo,	pH optimum
pH neutro,	neutral pH
reacción de doble desplazamiento,	double-displacement reaction
regresión no lineal,	non-linear regression
repetido,	duplicate (replicate)
representación de dobles inversos,	double reciprocal plot
representación lineal directa,	direct linear plot
representación secundaria,	replot / secondary plot
retención de la configuración,	retention of configuration
saturante,	saturating
suposición,	assumption
tendencia,	bias
(a) tiempo cero,	(at) zero time
valor aparente,	apparent value
variación,	variation

Verbos,	**Verbs**
comparar,	to compare
conseguir,	to accomplish
diseñar,	to design
escoger,	to choose, chose, chosen
requerir/necesitar,	to require
trazar (gráfico)	to plot

Academic Word List - General Terms

The following terms are part of the academic language of science, yet are not specific to science. It is necessary to understand these terms if one is to read and understand science literature. For more information on academic language: http://www.csun.edu/science/ref/language/index.html

achieve	corresponding	instance	relevant
acquisition	criteria	interpretation	required
alternative	data	journal	research
analysis	deduction	maintenance	resources
approach	demonstrate	method	response
area	derived	perceived	role
aspects	distribution	percent	section
assessment	dominant	period	select
assume	elements	positive	significant
authority	equation	potential	similar
available	estimate	previous	source
benefit	evaluation	primary	specific
circumstances	factors	principle	strategies
comments	features	procedure	structure
components	final	process	theory
concept	function	range	transfer
consistent	initial	region	variables

ANEXO 5: EJEMPLOS DEL TRABAJO DE ALUMNOS EN EL BLOQUE 3

1 Introducción y Objetivos

La lisozima o muramidasa (péptidoglicano N-acetil-muramil hidrolasa, EC 3.2.1.17) se aplica a diferentes componentes de la familia de enzimas que pueden catalizar la hidrólisis de enlaces glicosídicos β (1-4) de los polisacáridos de la pared bacteriana. Esta enzima se localiza en numerosas secreciones humanas como saliva, lágrimas, sudor y plasma sanguíneo; así como en otros vertebrados, invertebrados, virus plantas y bacterias. La estructura primaria de las diferentes lisozimas puede ser diferente pero todas ellas son proteínas globulares constituidas por una sola cadena polipeptídica y comparten una serie de características como: ser básicas (pI=10.5-11.0), tener una Mr baja (entre 14000 y 30000 Da), ser estables a pH ácido y presentar actividad lítica frente a bacterias Gram positivas como *Micrococcus lysodeikticus* (que usaremos para determinar la actividad de la enzima) [1] [2]

El objetivo principal de esta purificación es, a través de la recopilación de resultados, proponer el mejor método de purificación para la lisozima de clara de huevo de gallina, así como la introducción de pasos intermedios en el proceso de purificación realizado o la eliminación de alguno de ello que sea ineficaz.

2 Abstract

Hen egg-white lysozyme can hydrolyse β (1-4) glycosidic links of bacterial walls polysaccharides. In order to propose an efficient purification method we are going to purificate lysozyme following a concrete purification scheme. First, hen egg-white is exposed to an acid treatment followed by a thermal treatment so other proteins can be denaturalised without affecting lysozyme. The second step of the purification consists on chromatographic studies (ionic exchange chromatography using Amberlita CG-50, and molecular exclusion chromatography using Sephadex G-75), the comparison of the results will show the best way to purificate it. Once purification process is finished it will be determined activity and quantity of lysozyme in the different chromatographic fractions in order to stablish the purification grade of lysozyme and yield of the process. The chromatographic fractions will be analysed by PAGE-SDS electrophoresis in polyacrylamide gels to determine the protein composition of each fraction and to prove that the purification has been ended successfully. With all this information it will be proposed a purification method suggesting to add new steps on the scheme or to eliminate others.

3 Materiales y Métodos

3.1 Tratamiento ácido y térmico.

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3 Materiales y Métodos

3.1 Tratamiento ácido y térmico.

ABSTRACT

A purification treatment was set for egg white Lysozyme in order to aislate the protein from the rest of the albumen. Said purification involved an acid treatment followed by a thermic treatment, finishing with an Ionic Exchange Chromatography. The purification process was then registered and discussed. Comparisons were made between our purification process and the classes' overall purification procedure, and also between a Molecular Exclusion Chromatography based purification. Finally, a proposal was made to suggest a better purification treatment than the one employed.

Introducción

El objetivo de este estudio será el de elaborar una propuesta para aislar la lisozima a partir de la clara de huevo, o albumen. Para conseguir dicho objetivo se tendrá que tener en cuenta tantos las

actividad bacteriolítica (1, 4). Si aprovechamos estas características de la lisozima en diferentes tratamientos podremos realizar una purificación fiable.

Normalmente en el tratamiento de enzimas se

Filiación: Laboratorio de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, Universidad Complutense de Madrid.

Abstract The experiment was made from a hen egg white, to purificate the lysozyme which it contains. First, it was isolated thanks of an acid treatement. Secondly, it was subdues to a thermal treatment. Afterwards, it was separated with an ion exchange chromatography (Amberlita CG-50). The concentration was calculated using Bradford method, and, the activity was determinated using an enzyme essay. Futhermore, it was made an electrophoresis in SDS-PAGE. The purification at the end was 5,4 times.

1. Introducción

El nombre lisozima o muramidasa (peptidoglicano N-acetil-muramil hidrolada. EC 3.2.1.17) se aplica a los

Para ello se procederá a su purificación siguiendo un método determinado y después se analizarán los

El objetivo de esta práctica es realizar una propuesta razonada para aislar la lisozima a partir de la clara de huevo de gallina.

Abstract

The egg white lysozyme is a basic enzyme which function is to hydrolyze the cell walls of bacteria. It is a globular protein with a low molecular weight. The lysozyme is stable at both acid pH and high temperatures. Using this characteristics, the main goal is to propose a method to isolate and purify this enzyme.

Material y métodos

ABSTRACT: *the study describes the process for purification of lysozyme from hen ("Gallus gallus domestic") eggs [1]. Through different procediments, we isolated this protein and determinated the throughput of process. The crude egg extract was someted on acid treatment for denature the contaminant proteins, except lysozyme. After that, the simple was passed on a termic treatment for the same target. We applied two chromatographic technicals: cation exchanger Streamline chromatography and exclusion molecular chromatography. The employ of different chromatographic technicals will be useful to comparative aspects. So, it will be proposed an alternative method for purification of lysozyme. Throughput and efficiency of this process will be calculatd using diverse methods: activity essays, method to determinate protein concentration (method of Bradford) and an electrophoretic process. In the activity essay, we used bacterial wall extracts of from "Mycrococcus luteus" and measured the abs (450nm) to calculate the activity of each fractions (UAL). In the Bradford´s method, we used a pattern straight of BSA with known concentrations. Finally, we emploied an electrophoresis for distinguish the presence or absence of lysozyme in each phase of purification (E1, E2 and E3).*

INTRODUCCIÓN

El estudio desarrollado a continuación presenta como objetivo principal la realización de una propuesta alternativa de alta eficacia para la purificación de la lisozima de clara de huevo de gallina a través de argumentos razonados y comparaciones experimentales.

La lisozima, también conocida como muramidasa, hace referencia a un grupo de enzimas que catalizan la hidrólisis de enlaces glicosídicos de tipo beta(1-4) entre polisacáridos de N-acetilglucosamina y N-acetilmurámico en las paredes baterianas (Gram positivas). Las lisozimas pueden ser encontradas en secreciones humanas (saliva, lágrimas, mucus) como en sustancias naturales como la clara de huevo. En este proceso emplearemos la clara de huevo procedente de gallina (*Gallus gallus domestic) [1]* como fuente biólogica para la purificación. La clara de huevo contiene minerales, vitaminas, glucosa y más de 20 proteínas diferentes, siendo la ovoalbúmina la más abundante, mientras que la ovotransferrina y la lisozima se producen sólo en un 10-12% y 1-2% respectivamente. La acción de la lisozima frente a las

ANEXO 6: EJEMPLOS DEL TRABAJO DE ALUMNOS EN EL BLOQUE 4

ABSTRACT

Almond β-glucosidase catalyses the hydrolysis of *p*-nitrophenyl-β-D-glucoside releasing β-D-glucose and *p*-nitrophenol. Here, we describe a study in order to determinate: the kinetic parameters, the effect of the temperature in the catalysis and the effect of glucose and δ-gluconolactone as inhibitors of the enzyme. We obtain Km 2.17± 0.14mM, V_{max} 14.31 ± 0.45 μM/min and a loss of the enzimatic activity from 70ºC. Besides, glucose and δ-gluconolactone are competitive inhibitors of β-glucosidase. These results suggest that this enzyme follows a "ping-pong" mechanism, in which *p*-nitrophenyl-β-D-glucoside enters first, then the reaction is held with the formation of a ternary complex; after this, *p*-nitrophenol is released; then, water enters and, finally, glucose is liberated.

Great

Abstract

The kinetic characterization of a β-glucosidase from *Prunus dulcis* is described. The studies were carried out at 40ºC and at pH 5. The β-glucosidase hydrolyzed p-nitrophenyl-β-D-glucopyranoside (pNPG), exhibiting a Km of (2.17 ± 0.51) mM and a Vmax of (14.31 ± 0.98) μM · min^{-1}. The enzyme showed an optimal activity at 55ºC and was fairly stable up to 50ºC. Therefore, an optimal assay temperature of 50ºC was proposed. The activation energy of the reaction was 25.7 kJ · mol^{-1}. δ-Gluconolactone and glucose were shown to inhibit the aforementioned β-glucosidase in different ways : while δ-gluconolactone acted as a competitive inhibitor with a Kic of 0.203 mM, glucose was found to be a mixed inhibitor of the enzyme, with a Kic of 380 mM and a Kiu of 1060 mM. δ-Gluconolactone inhibition studies provided structural information on the transition state of the enzyme. Nevertheless, the obtained results regarding glucose do not match the previously reported data on the inhibitory character of the monosaccharide: glucose is actually known to act as a competitive inhibitor of sweet almond β-glucosidase. Thereafter, the reaction's kinetic mechanism could not be determined. It would consequently be recommended to repeat the inhibition studies in order to obtain more consistent experimental results and to carry out pH studies with a view to submitting a comprehensible chemical mechanism and, consequently, a conclusive catalytic mechanism.

Well done!

18 de Diciembre, 2014

Abstract: **This study describes the enzymatic characterization of the beta-glucosidase *Prunus dulcis*, due to its great industrial and medical interest as it shows the hydrolysis reaction of β-O-glycosidic bonds and transglycosylation. A steady-state kinetic analysis showed a Km of 1.66 mM, Vmax of 12.16 mM/min, kcat of 3974 min^{-1} and a catalytic efficiency (kcat/Km) of 2394 $mM^{-1}min^{-1}$. The optimal assay conditions have been achieved at a concentration of 3.1 nM, a pH of 5, a temperature range of 40-60^{0}C, a range of concentrations of 0.5-20 mM pNPG and test time 10 minutes. Glucose and glucono-δ-lactone inhibited the β-glucosidase competitively with Ki values of 494 mM and 0.1 mM, respectively. These results suggest a Ping Pong Bi Bi mechanism of the beta-glucosidase of *Prunus dulcis*, in which pNPG binds first followed by the dissociates of pNP and the binding of water followed by glucose release. All results are consistent with those proposed by other researchers for beta-glucosidase from other organisms, suggesting that the catalysis mechanism of beta-glucosidase is conserved across different organisms.**

Laboratorio de Bioquímica y Biología Molecular I, Universidad Complutense de Madrid

Diciembre 2014

Abstract: The enzymatic characterization of β-glucosidase from *Prunus dulcis* almond has been carried out in a series of steps based on its catalytic properties: assay conditions standardization, kinetic parameters determination, effect of temperature on the catalytic activity of *P.dulcis* β-glucosidase and inhibition studies by glucose as a forward reaction product and δ-gluconolactone as a transition state analog. It was possible to conclude that *P.dulcis* β-glucosidase showed a K_M=1.57±0.26 mM and a V_{max}=15.56±0.60 μM/min for *p*-nitrophenol-β-D-glucoside, which seem to agree with the kinetic parameters of other β-glucosidases from plants. In addition to this, both glucose and δ-gluconolactone behaved as competitive inhibitors (K_{CI} = 494 mM and 96 μM, respectively), which allowed to confirm δ-gluconolactone as a transition state analog (chemical mechanism described) and glucose to be the last product to came out of the β-glucosidase active site, consequently resulting in a cripto ping-pong kinetic mechanism.

Laboratorio de bioquímica y biología molecular; dpto. de bioquímica Universidad Complutense de Madrid.

ABSTRACT

The kinetic mechanism from β-glucosidase has a high interest. For its analysis it was used β-glucosidase from almonds that hydrolyzed p-NPG getting as products p-NP and glucose. The proposed study includes standardization of the assay conditions; macroscopic kinetic parameters determination of the enzyme; effect of the temperature in the kinetic parameters; inhibition study of the catalysis of β-glucosidase by glucose and δ-gluconolactone; as well as a proposal of a model for the kinetic mechanism of the reaction catalyzed by β-glucosidase.

Conclusions: After doing all the assays, it's concluded that the β-glucosidase presents a ping-pong kinetic mechanism. (there are more conclusions: e.g. kinetic parameters, Km, Smax and so on

Facultad de Química. Universidad Complutense de Madrid. 2014.

ABSTRACT

B-glucosidases are enzymes that hydrolyze O-β-glycosidic bond at the nonreducing terminal end of short chain oligosaccharides, disaccharides and aryl- or alkyl-β-D-glucoside, releasing β-D-glucose. Here, it has been carried out the kinetic characterization of the enzyme, using the reaction between p-nitrophenyl-β-D-glucoside (*p*NPG) and β-glucosidase to produce glucose and p-nitrophenol (*p*NP). Previous studies have been done to determine ~~which were~~ the optimal assay conditions for the enzyme and once ~~it was~~ they were set up, assays were performed to determine kinetic parameters, to check the temperature effect and check the effect of some inhibitors. As of the results of these assays, it was possible to determine that the β -glucosidase has a ping-pong catalytic mechanism. Following this mechanism, it can be stated that the first substrate that comes in is *p*NPG, and the first product that comes out is *p*NP. The enzyme forms an intermediate state, which reacts with H_2O and leads to the formation of glucose, leaving free enzyme again. Some conclusions are missing.

ANEXO 7: POSTERS DE LOS ALUMNOS

KINETIC CHARACTERIZATION OF SWEET ALMOND β-GLUCOSIDASE

N, GARCÍA DOMÉNECH AND P, GARCÍA SOCUÉLLAMOS

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INTRODUCTION

β-glucosidase is a group of enzymes which are able to hydrolyze O- or S-glycosidic bonds between a carbohydrate and another carbohydrate or an aglycone. While we were performing these experiments, we used the same assay but changing some of the conditions depending on which parameter we want to measure. Firstly, we had established the enzyme concentration that we were going to use in the rest of the assays, followed by the determination of the kinetic parameters such as Km or kcat. Secondly, we had studied the effect of the temperature in the catalytic action of the β-glucosidase. And finally, we had performed an inhibition study to establish the kinetic mechanism. With the data obtained with this studies and the information provided by bibliography we could reach the final objective which is to determinate the catalytic mechanism of this β-glucosidase.

MATERIAL AND METHODS

- β-glucosidase (*Prunus dulcis*).(*SIGMA*) (170nM)
- *P*-nitrophenol (*p*NP) (*FLUKA*) (25mM)
- *P*-nitrophenyl-ß-D-glucoside (*p*NPG) (*FLUKA*) (50mM)
- Glucose (*FLUKA*) (2M)
- δ-gluconolactone (*FLUKA*) (20mM)
- Citrate sodium buffer 100mM, pH 5.0.
- NaOH (0.2M)

0.9 ml buffer + pNPG* —(5 min, T (ºC))→ + 0.1ml enzyme —(t (min), T (ºC))→ +1.5ml NaOH → A_{410}

*In the inhibition studies, we also add the predeterminate inhibitor concentration.

RESULTS

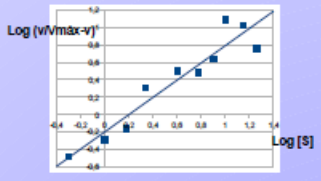
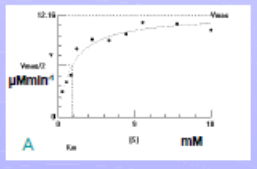


Figure 1. Hill's plot.



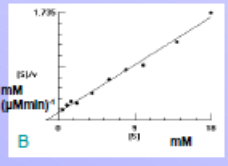
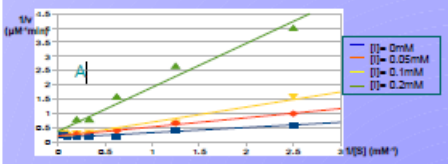


Figure 3. Michaelis Menten (A) and Hanes-Woolf (B) plots.

Figure 2. Arrhenius's plot.

Table 1. Summary of the results obtained using an enzyme optimum concentration of 3nM.

Km (mM)	Vmax (μM/min)	Kcat (min^{-1})	Catalytic efficiency ($min^{-1}mM^{-1}$)	Hill coeficient	Activation energy (kJ)
1.61	12.16	4063.33	2520	1	21.68



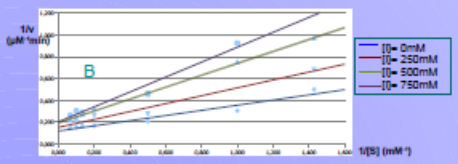


Figure 4. Inhibition studies with δ-gluconolactone (A) and glucose (B).

Inhibitor	Kic (mM)	Kiu (mM)
δ-gluconolactone	0.05	——
Glucose	380	1060

Table 2. Inhibition constants. Kic= competitive constant. Kiu= acompetitive constant.

Glucose (Product of the reaction)

δ-gluconolactone (Transition state analog)

CONCLUSION

With the data obtained with our experiments and with bibliography we have determinated that the catalytic mechanism of β-glucosidase of sweet almond emulsin is an unitbi crypto pin-pong mechanism in wich there are two catalytic stages with an acid-base catalytic mechanism in each one. The first product released is pNP and a covalent intermediate is formed between the glucose and the enzyme. In a second stage, water hydrolyzes the bond between the enzyme and the glucose and glucose is released. To check if the kinetic mechanism is correct we should do an additional inhibitor study with the other product, pNP.

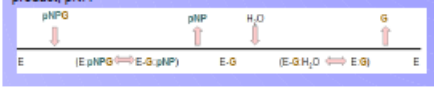


Figure 5. Cleland representation of the β-glucosidase catalysis.

KINETIC CHARACTERIZATION OF β-GLUCOSIDASE OF ALMOND

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Curso 2014-2015

INTRODUCTION

β-glucosidases (E.C. 3.2.1.21) are enzymes that hydrolyze glycosidic bonds of nonreducing terminal glucosyl residues from glycosides and oligosaccharides, releasing β-D-glucose as product. The currently used classification is based on the sequence and the folding process of these enzymes, dividing the β-hydrolases in lots of families, of which the family 1 and 3 contains the β-glucosidase. Biological sources of β-glycosidases are very varius: there are archaea, plants, mammals, fungy, bacterial and yeasts enzymes. In fungi and bacteria, the β-glucosidases are part of multienzymatic complexes called cellulases. These enzymes are used in food, paper, pharmaceutical, chemical and textile industries, and also in biomass production.

An enzymatic assay will be optimized to determine the activity and the kinetic parameters of the β-glucosidase of almond in steady state, in order to propose the catalytic mechanism of the enzyme. The temperature effects and a product and transition state analog inhibitions will be studied.

MATERIALS AND METHODS

1. **pNP standard calibration curve.**
2. **Standardization of assay conditions:** optimal [enzyme], Km, optimal time of reaction.
3. **Determination of kinetics parameters for pNPG** with conditions of 40ºC, optimal [enzyme], optimal time and different pNPG concentrations. **Km, Vmáx, Kcat, Ecat.**
4. **Effect of temperature in catalysis:** 25ºC, 35ºC, 45ºC, 55ºC, 60ºC.
5. **Effect of inhibitors in catalysis:** glucose (product of reaction, δ-gluconolactone (transition state analogue).

Materials: β-glucosidase of almond, p-nitrofenil-glucose (pNPG) were obtained from FLUKA, and phosphate buffers, NaOH, HCl, citric acid from PANREAC.

RESULTS

Standarization of the assay conditions: At first, a pNP standard curve was built, and its equation was calculated: Abs_{410nm}=0,005(pNP mM)+0,040, r^2 =0,988. Then, the standard conditions were calculated: optimal concentration of enzyme (Figure 1), Km appearance (Figure 2), linearity over time (Figure 3), and optimal temperature (Figure 4).

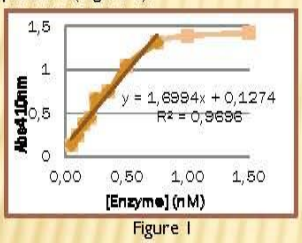

Figure 1

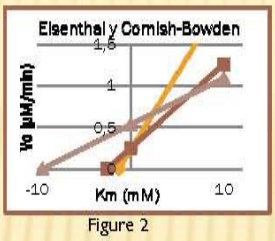

Figure 2

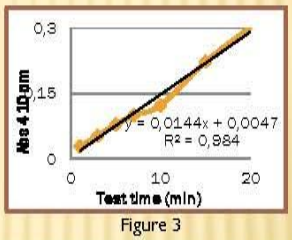

Figure 3

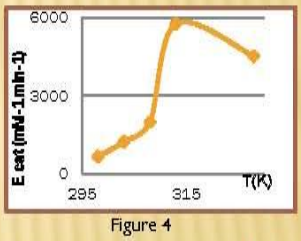

Figure 4

Kinetic parameters:
They were calculated by diferent representations built with *Hyperbolic Regression* (Figure 5), showing a value of Km=4.3690±4.406 mM and a Vmax of 6.319± 2.571 μM/min.

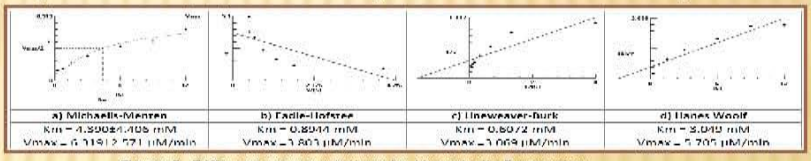

Figure 5. Different representations by Hyperbolic Regression

Inhibition studies by glucose and δ-gluconolactone:
The behavior of the inhibitors was determined by studying the kinetic parameters of the enzyme for pNPG with 4 different concentrations of each inhibitor. The KEI of glucose and δ-gluconolactone was calculated by a secondary representation (figure 6 and 7), their values were 139 mM for the glucose and 0.032 mM for δ-gluconolactone.

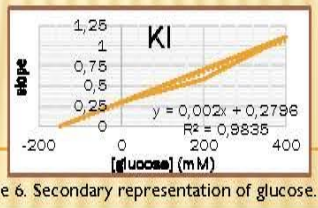

Figure 6. Secondary representation of glucose.

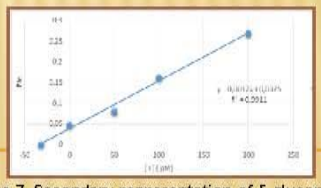
Figure 7. Secondary representation of δ-gluconolactone

CONCLUSION

To optimize the assay, the optimal concentration of enzyme (0.25 nM), the optimal testing time (10 min), the approximate Km (2,40 mM) and the optimum temperature (42°C) were determined. The inhibiting behavior of glucose and δ-gluconolactone was studied and it showed a competitive inhibition with KEIs of 139 and 0,032 mM respectively. Finally, a Cleland scheme was developed (figure 8).

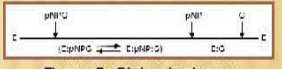
Figure 8. Cleland scheme

BIBLIOGRAPHY

- Henrissat, B.; Davies, G., Structural and sequence-based classification of glycoside hydrolases. *Current Opinion in Structural Biology* 1997, 7, 637-644.
- White, A.; Rose, D. R., Mechanism of catalysis by retaining beta-glycosyl hydrolases. *Current Opinion in Structural Biology* 1997, 7, 645-651.
- Itohnashida, T.; Hiraiwa, M.; Uda, Y., Purification and properties of beta-D-glucosidase(linamarase) from the butter bean, Phaseolus lunatus. Journal of *Biochemistry* 1987, 101,847-854.
- Bhat, M. K.; Bhat, S., Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances* 1997, 15, 583-620.

ENZYMATIC CHARACTERIZATION OF β-GLUCOSIDASE FROM *Prunus dulcis*

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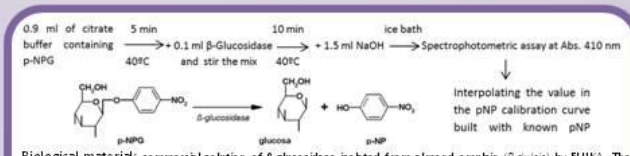
INTRODUCTION

β-glucosidases are a largely distributed group of enzymes that play an important role in nature, for example in cellulose multienzymatic system and chemical defense mechanisms in plants [1]. As a result, these enzymes are currently applied to the food, textile and biotechnological industries [2]. The enzymatic characterization of β-glucosidase from *Prunus dulcis* almond has been carried out in this study in a series of steps based on its catalytic properties: assay conditions standardization, kinetic parameters determination, effect of temperature on the catalytic activity of *P.dulcis* β-glucosidase and inhibition studies by glucose as a forward reaction product and δ-gluconolactone as a transition state analog.

MATERIALS AND METHODS



Biological material: commercial solution of β-glucosidase isolated from almond emulsin (*P.dulcis*) by FLUKA. The enzyme is a homodimer (Mr=67.000 per subunit). Chemicals: p-nitrophenil-β-D-glucoside (pNPG), p-nitrophenol (pNP), glucose and δ-gluconolactone (all from FLUKA). The reaction medium was a 100 mM, pH 5.0 sodium citrate buffer.

RESULTS

1. Standardization

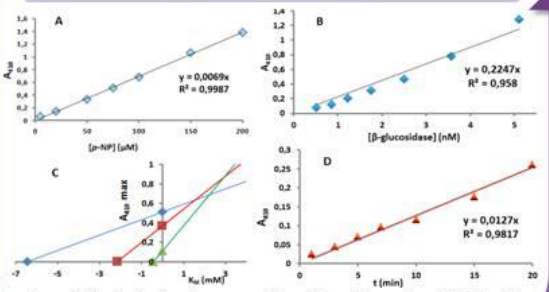
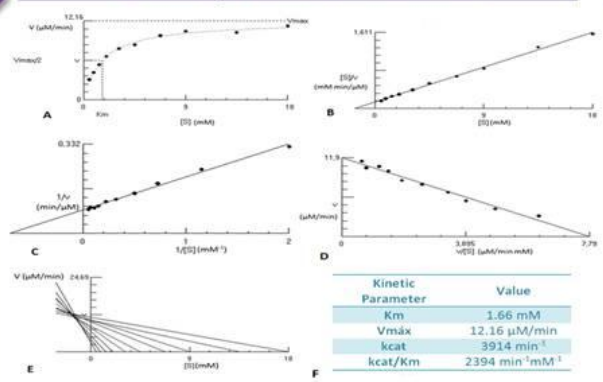


Figure 1. Standardization of assay conditions. A. Standard curve for p-NP. B. Optimal concentration of β-glucosidase. C. Eishental and Cornish-Bowden plot. D. Time linearity.

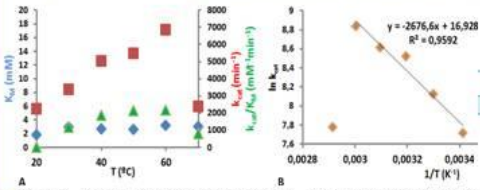
2. Kinetic parameters determination



Kinetic Parameter	Value
Km	1.66 mM
Vmáx	12.16 μM/min
kcat	3914 min^{-1}
kcat/Km	2394 $min^{-1}mM^{-1}$

Figure 2. Kinetic parameters determination. A.Michaelis-Menten plot. B. Hanes-Wolf plot. C. Lineweaver-Burk plot. D. Eadie-Hofstee plot. E. Hyperbola adjustment. F. Table showing the calculated kinetics parameters.

3. Effect of temperature



Ea (kJ/mol)	Q10
22. 25	1.36

Figure 3. Effect of temperature on catalysis. A. Variation of the kinetic parameters with temperature. B. Linearization of Arrhenius equation. C. Table showing the calculated activation energy and Q10 factor.

4. Inhibition studies

Results show that both glucose and δ-gluconolactone behave as competitive inhibitors, with K_{ci} values of 494 mM and 96 μM respectively.

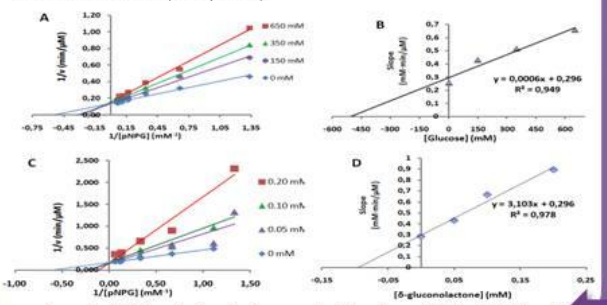


Figure 3. Inhibition studies. A. Lineweaver-Burk for glucose inhibition. B. Replot for glucose inhibition. C. Lineweaver-Burk for δ-gluconolactone inhibition. B. Replot for δ-gluconolactone inhibition.

CONCLUSIONS

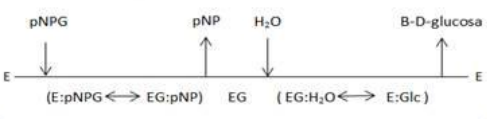
1. The optimal temperature of β-glucosidase from *P.dulcis* is approximately 60 ºC but the assay is better carried out at 40 ºC in order to avoid the active site denaturation (which in this case began at 60 ºC).

2. The effect of temperature on catalysis suggest that K_M does not depend on temperature while k_{cat} does.

3. δ-gluconolactone is confirmed to behave as a transition state analog while glucose turns out to be the last product to came out of the β-glucosidase active site. These patterns of inhibition are consistent with a cripto ping-pong kinetic mechanism.

4.Studies from other authors suggest a double displacement mechanism with retention of configuration [3].

Cleland notation:



References:

[1] Butters, T.D. (2007) Gaucher disease. Curr Opin Chem Biol, 11, 412-418.

[2] Baldrian, P.; Gabriel, J.; (2003) Lignocellulose degradation by *Pleurotes Ostreatus* in the presence of cadmium. FEMS Microbiol Lett, 220, 235-40.

[3] Rye, C.S.; Withers, S.G. (2000). Glycosidase mechanism. Curr Opin Chem Biol, 4, 573-580.

β-GLUCOSIDASE FROM SWEET ALMOND

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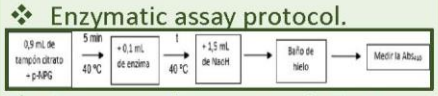
INTRODUCTION

An enzymatic caracterization for β-gluosidase from sweet almond was approached in order to obtain its kinetic and catalytic mechanism. B-glucosidase catalyzes S-glicoside or O-glicoside bonds. In addition, the enzyme has displayed a major interest in food, farmacological and textile industries.

METHODOLOGY

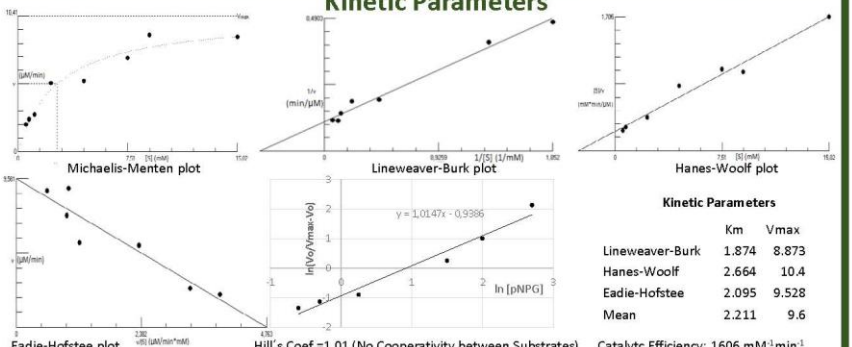
- Enzymatic assay protocol.



- Assay conditions standarization.
- Kinetic parameters determination.
- Study of the thermical effect during catalysis.
- Inhibition studies.

RESULTS

Kinetic Parameters



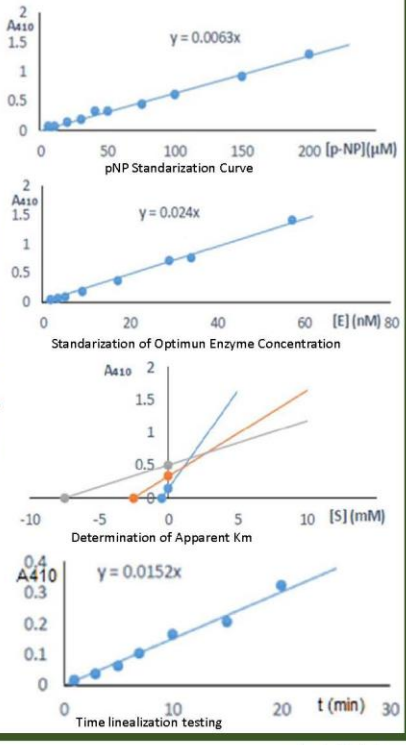
Kinetic Parameters

	Km	Vmax
Lineweaver-Burk	1.874	8.873
Hanes-Woolf	2.664	10.4
Eadie-Hofstee	2.095	9.528
Mean	2.211	9.6

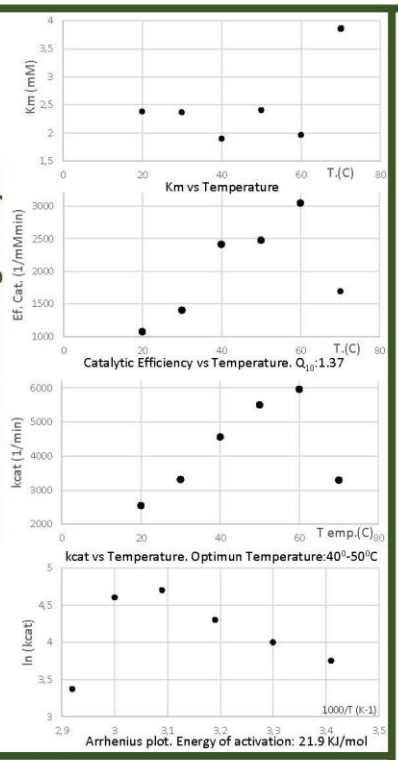
Catalytc Efficiency: 1606 $mM^{-1}min^{-1}$

kcat:3413 min^{-1}

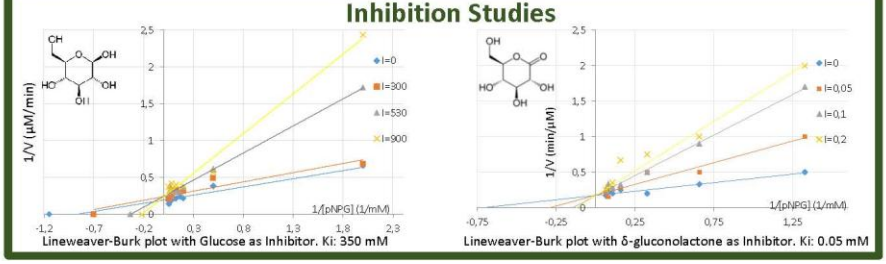
Study´s Standarization



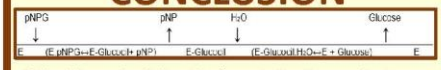
Thermical Effect during Catalysis



Inhibition Studies



CONCLUSION



Cleland diagram for β-glucosidase´s crypto Ping-Pong mechanism

The inhibition studies performed determine that an intermediate state in catalysis is necessary. This intermediate state requires that a flat triangular conformation is present in the anomeric carbon. Said conformation is complementary to β-glucosidase active site in contrast of the chair conformation known in glucose. Therefore, the catalysis will occur in two stages in which an intermediate covalent state takes place. pNP will be the first product to leave the active site and β-glucose will be the second one. β-glucosidase holds a crypto Ping Pong bisubstrate mechanism in which water provoques the intermediate state cleavage liberating the product from the enzyme.

Kinetic Characterization of β-Glucosidase from Almond

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1. INTRODUCTION

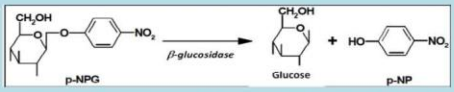
β-glucosidase (β-D-glucoside glucohidrolase, EC 3.2.1.21) is an enzyme which catalyzes the reaction of hydrolysis of O-β-glicosidic bond in the terminal non reductor end of different carbohidrates. In this reaction β-D-glucose is released. This enzyme can be found in many biological sources such as bacteria, fungi, plants, mammals... where it carries out many physiological functions [1], for instance, defense mechanism and growth in plants. In human beings, its deficiency causes the Gaucher disease [2]. The most accepted classification of glicoside hidrolases is based on similarities of sequence and folding and it establishes more than 100 families [3]. β-glucosidase is mainly found in 1st and 3rd family. This enzyme is very important in textile, food and biotechnological industries.

The final objective of this project will be the proposal of a model for de catalytic mechanism of the almond β-glucosidase.

2. RESULTS

1. Standardization of the Enzymatic Assays

In order to standardize the assay conditions of almond β-glucosidase, *p*NPG has been used as substrate.

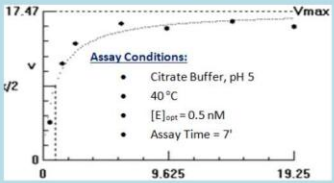


Different experiments were carried out and the results are shown in the next figure:

Parameter	Value	Unit
$[Enzyme]_{optimal}$	0.5	nM
Range of [Substrate]	0.5-19.25	mM
Assay Time	7	min
Temperature	40	ºC
Assay Volume	1	mL

When the experimental conditions were established, Michaelis-Menten assumptions were taken into account.

2. Determination of Kinetic Parameters



In order to determine the macroscopic kinetic parameters of the enzyme it is necessary to analyze the dependence of the initial velocity of catalysis with substrate concentration.

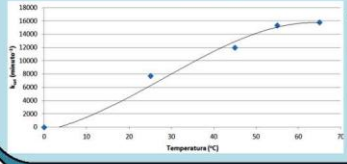
For this reason, a PC program with the least squares fitting technique to the hyperbole was used.

Parameter	Value	Unit
Maximum Velocity	17.47 ± 1.967	μM/min
K_m	0.9334 ± 0.5316	mM
K_{cat}	34940	min^{-1}
K_{cat}/K_m	37449	$mM^{-1} \cdot min^{-1}$

The first figure shows the Michaelis-Menten plot and the assay conditions used for calculate the kinetic parameters which appear in the table.

3. Effects of Temperature in the Catalysis

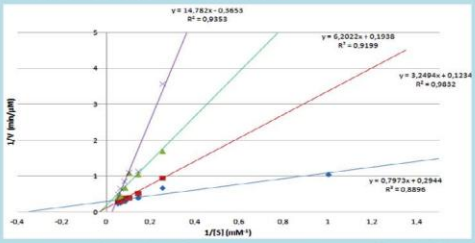
The effect of temperature was analyzed determining the kinetic parameters of the enzyme at different temperatures.



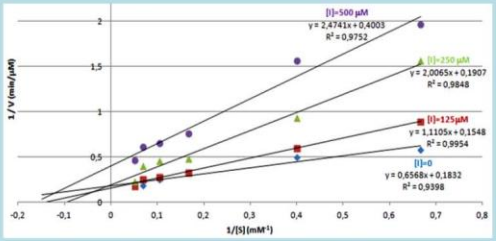
The observed maximum of the plot corresponds to 65 ºC, which is the optimum temperature. The denaturation temperature could not be determined with the experimental data.

4. Reversible Inhibition Study

The inhibitory behavior of the glucose and the δ –gluconolactone was determined by analyzing the kinetic parameters of the enzyme for the *p*NPG in the presence of 4 different concentrations of inhibitor.

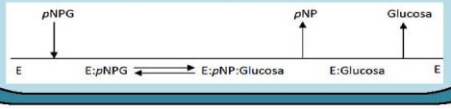


The glucose inhibition figure shows a competitive inhibition with a K_i = 142.70 mM.



The δ–gluconolactone inhibition figure shows a typical competitive inhibition and its K_i was 107.98 μM.

Due to glucose shows a competitive inhibition, it is the last product in leaving the catalytic centre. For this reason, the following kinetic mechanism is proposed:



3. CONCLUSIONS

- The temperature in which the enzyme activity is maximum is 65 ºC.
- The enzyme posses a sequential-ordered Uni-Bi kinetic mechanism.
- δ-gluconolactone is proved to be a great transition state analogue and inhibits better than the glucose.
- To continue the investigations, experiments to analyze the effect of the pH on the catalysis could be carried out.

[1]: Bhatia, Y.; Mishra, S. y Bisaria, V. S.(2002); *"Microbial b-glucosidases: Cloning, properties, and applications"*. *Critical Reviews in Biotechnology*; Vol. 22, páginas: 375-407. [2]: Grabowski, G. A.; Gatt, S. y Horowitz, M. (1990); *"Acid b-glucosidase: enzymology and molecular biology of Gaucher disease"*. *Critical Reviews in Biochemistry and Molecular Biology*; Vol. 25, páginas: 385-414. [3]: Henrissat, B. y Davies, G.J. (1997); *"Structural and sequence-based classification of glycoside hydrolases"*. *Current Opinion in Structural Biology*; páginas: 637-644.

KINETIC CHARACTERIZATION OF β-GLUCOSIDASE

Cristina Balbás, Rocío Bartolomé, Alberto Carnicero y Laura Fernández. Laboratorio de Bioquímica y Biología molecular (UCM) 2015

Introduction

β-glucosidases are enzymes that hydrolyze glycosidic bonds to release non-reducing terminal glucosyl residues from glycosides and oligosaccharides. In this study an almond beta-glucosidase was analyzed to determine its kinetic parameters, and the effect of several environmental factors such as temperature and competitive inhibitors on enzymatic catalysis.

Methods

1. ENZYMATIC ASSAY

Figure 1. Reaction scheme followed in all the enzymatic assays for the standarization of optimal conditions and the determination of the kinetic parameters. This scheme was also followed at different temperaturas so as to determine the effect of temperature in enzymatic catalysis

2. STUDIES OF DIFFERENT INHIBITORS

Reversible inhibition assays were performed using δ-gluconolactone, a transition state analogue, and glucose, a product of the catalysis of the enzyme.

Results and discussion

The optimal conditions determined were:
-[E]: 0.45 nM
-Time: 10 min

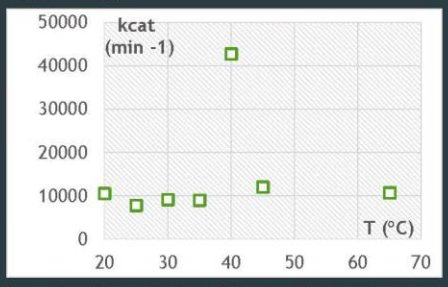


Figure 2. The evolution of Kcat with temperature.

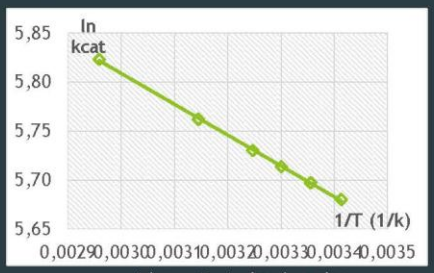


Figure 3. Arrhenius plot.

The studies show that the optimun temperature is 40°C, after which denaturing occurs (Figure 2). It is also possible to determine the activation energy, Ea = 2617 J/mol (Figure 3).

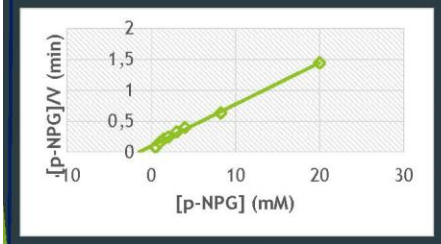


Figure 4. Hanes-Woolf plot

Table 1. Kinetic parameters

	Michaelis	Dobles inversas	Eadie-Hostee	Hanes-Woolf
Km (mM)	1,2	1,34	1,14	1,33
Kcat (min^{-1}) (Ecuación 3)	33500	33550	33000	37025
Vmax (μM/min)	13,4	13,42	13,2	14,81
E. catalítica ($mM^{-1} s^{-1}$) (Ecuación 4)	465,28	417,29	482,46	463,97

After the analysis of the kinetic parameters of each plot, is possible to deduce that the closest to reality is Hannes-Woolf although the rest are mostly similar (Table 1).

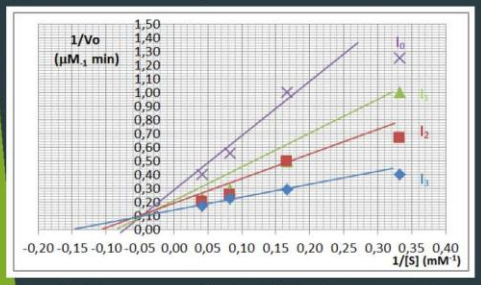


Figure 5. Lineaweaver-Burk plot for δ-gluconolactone inhibition.

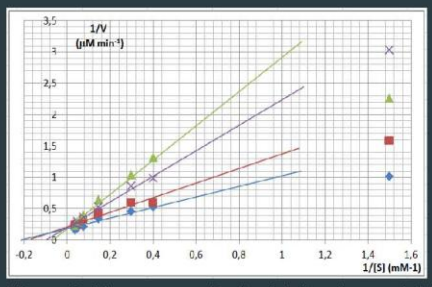


Figure 6. Lineaweaver-Burk plot for glucose inhibition

Analyzing figures 5 and 6, it is displayed that both δ-gluconolactone and glucose deal competitive inhibition. This is known by seeing that data has different slopes but the same incertept at Y axis. Dixon plot was used to determine Ki (figure not shown).

Conclusion

The results obtained were (approximately) a Km of 1,25 mM, an Vmax of 13,5 μM/min, a Kcat of 35000 min^{-1}, and a catalytic efficiency constant of 460 $mM^{-1} s^{-1}$. The optimal temperature rounds 45°C and both δ-gluconolactone and glucose act as competitive inhibitors, with a Ki of 0,1 and 250 mM, respectively. The proposed catalytic mechanism was uni-bi. By analyzing the results of the glucose inhibition, it is provided the Cleland scheme (figure 7).

Figure 7. Cleland scheme for β-glucosidase.

β-glucosidase: catalytic mechanism

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Laboratory of Biochemistry and molecular biology I

1.Introduction

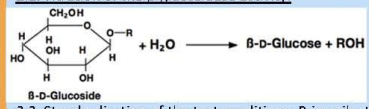
β-glucoside glucohydrolases, (EC 3.2.1.21), or also known as β-glucosidases, constitute an enzyme group within the glucosidases. β-glucosidases hydrolyze the O-β-glucosidic bond located at the non-reducting terminus of short chain oligosaccharides, disaccharides and aril- or alquil-β-D-glucosides; it produces β-D-glucose [1].

In bacteria and fungus, this enzyme is part of the multienzymatic systems denominated cellulases [2]. They are responsible for the glucose's degradation. In plants [3], the β-glucosidase gets involved in chemical defense mechanisms; activation of the fito-hormones' precursors; hydrolisis catalyzed of the oligosacarides resulting from the degradation of the cellular wall; and aglycones release. In mammalians, cytosolic β-glucosidases take part in the xenobiotics metabolism. In fact, in humans the lisosomal acid β-glucosidase deficiency, that hidrolyzes glucosilceramides, origines the Gaucher disease[4,5] .

The standardization of the assay conditions have been accomplised, as the determination of the kinetics parametres for the substrate (pNPG). It has also been analyzed the effect of the

2. Methods

2.1. Titration of the β-glucosidase activity.



2.2. Standardization of the test conditions. Primarily, the assay conditions have been established, including:
a)Incubation time
b)Optimal enzyme concentration for the assay
c)Substrate concentration (Km*)
Subsequently, it has been ascertained both time and enzyme concentration's linearity in order to finally assess the percentage of transformed susbtrate ({[ ])/({[ ]) 100) and the molar ratio between substrate and enzyme ({[*]as/[ ]as).*

2.3. Determination of kinetic parameters. To determine the macroscopic kinetic parameters, the velocity dependence with substrate concentration has been analyzed. Therefore, assays have been carried under the experimental conditions previously established, including fixed [E] and varying [S].

2.4. Temperature effect in catalysis. It was analyzed by determining the kinetic parameters using incubations at different temperatures. The activation energy can be reckoned by plotting ln Kcat vs 1/T (K-1), using Arrhenius ecuation: Kcat=A x e^{-Ea/RT). It also can be calculated Q10 coeficient: 10[[=(_2/ _1)]]^(10/({ 2− 1))).

2.5. Reversible inhibition studies. The inhibitor behaviour of glucose, hydrolisys product, and δ-glucolactone, transition' state analog, have been analyzed by studying the kinetic parameters in the presence of 4 inhibitor concentrations.

3. Results

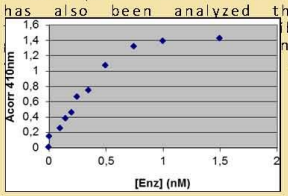


Figure 1. Standardization. Linearity of the enzyme concentration. Optimum [E] = 0,25 nM.

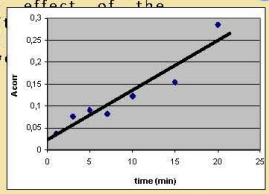


Figure 2. Standardization. Linearity of A_{410} within time. Optimal incubation time: 10 min.

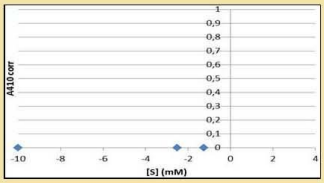


Figure 3. Standardization. Cornish-Bowden plot. It enables the calculation of Km* = 2,44 mM. Transformed substrate= 4%. Molar substrate/enzyme ratio = 2·10^6.

Table 1. Kinetic parameters of each plot.

Representación	Vmax (μM/min)	Km (mM)	Kcat (min^{-1})	Ef.Cat ($mM^{-1} min^{-1}$)
Dobles inversas	4.71	4	18840	4710
Eadie-Hofstee	4.25	3.23	17000	5120
Hanes-Woolf	4.34	3.4	17360	5106
Eisenthal y Cornish-Bowden	4	2.875	16000	5565
Hyperbolic Regression	4.185	3.287	16740	5092.8

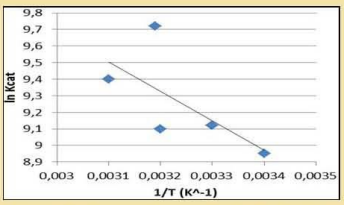


Figure 4. Temperature effect. Arrhenius plot: ln Kcat vs 1/T (K^-1). Q10 = 0,996. Ea = 16074 J/mol.

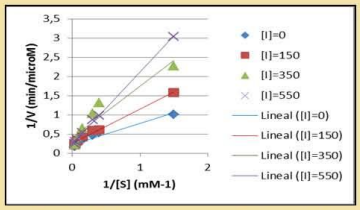


Figure 5. Inhibition. Lineweaver-Burk plot for various glucose concentrations. Different slopes, same origin: competitive inhibition.

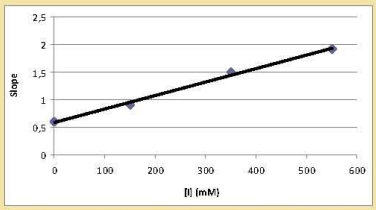


Figure 6. Inhibition. Dixon plot: slope vs inhibitor concentrations. Glucose Ki = 250 mM, not a good inhibitor.

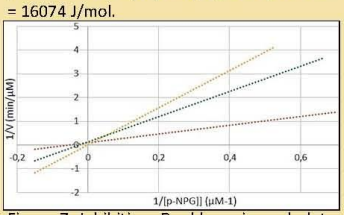


Figure 7. Inhibition. Double reciprocal plot for various δ-glucolactone concentrations. Competitive inhibition.

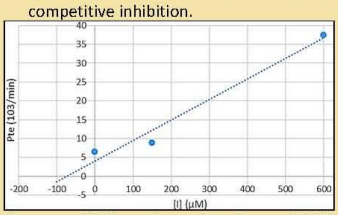


Figure 8. Inhibition. Dixon plot: slope vs inhibitor concentrations. δ-glucolactone Ki = 0,1 mM. Powerful inhibitor.

4. Conclusion

The collected data from Km and optimal temperature are reasonable and resemble those of *Olea europea*, whereas the inhibition data are similar to *Zea mays*.

β-glucosidase reaction using p-NPG it's uni-bi, as a ping-pong mechanism. Glucose is the last generated product, so it proves that the exerted inhibition is competitive.

Regarding the chemical mechanism, thanks to the pH studies is known that there are two Glu residues taking part into the catalysis: one as an acid and another as a base, allowing the creation of the glucosyl-enzyme intermediate that will lead to the releasing of glucose and free enzyme.

5. References

[1]. Mladenoska, I.; Grey, C.E.; Winkelhausen, E.; Kuzmanova, S.; Adlercreutz, P., Competition between transglycosylation and hydrolysis in almond beta-glucosidase-catalyzed conversión of p-nitrophenyl-bete-D-gluvoside in monophasic water/alcohol mixtures. *Biocatalysis and Biotransformation* **2007**, 25, 382-385.

[2]. Bhat, M.K.; Bhat, S., Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances* **1997**, 15, 583-620.

[3]. Morant, A.V.; Jorgensen, K.; Jorgensen, C.; Paquette, S.M.; Sanchez-Perez, R.; Moller, B. L.; Bak, S., Beta-glucosidases as detonators of plant chemical defense. *Phytochemistry* **2008**, 69, 1795-1813.

[4]. Grabowski, G.A.; Gatt, S.; Horowitz, M., Acid beta-glucosidase: enzymolohy and molecular biology of Gaucher disease. *Critical Reviews in Biochemistry and Molecular Biology* **1990**, 25, 385-414.

[5]. Butters, T.D., Gaucher disease. *Current Opinion in Chemical Biology* **2007**, 11, 412-418.

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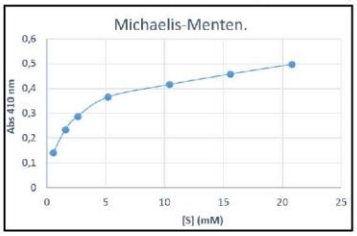
KINETIC STUDIES OF ALMOND β-GLUCOSIDASE

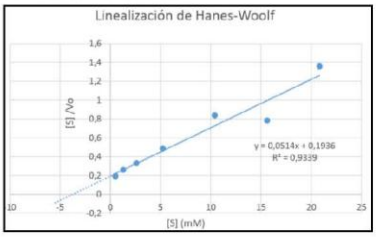
Introduction

β-glucosidase catalyzes the hydrolysis of terminal non-reducing residues in β-D-glucosides with release of glucose. These enzyme are important in many biotechnological processes. The application of β-glucosidases in industrial processes requires a detailed knowledge about them, specially their reaction mechanism. Almond β-glucosidase was characterized by studying an hydrolysis reaction with p-NPG as substrate, changing assay conditions.

Determination of kinetic parameters

The final enzymatic essay was made in a temperature of 40ºC, an optimal enzyme concentration of 0,2 nM and a range of substrate concentracions from 0,52 to 20,8 mM. The optimal time of reaction was 5 minutes. After making sure the kinetics followed a Michaellis-Menten model, these plots were obtained:

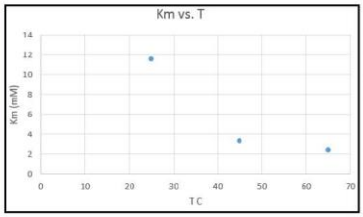


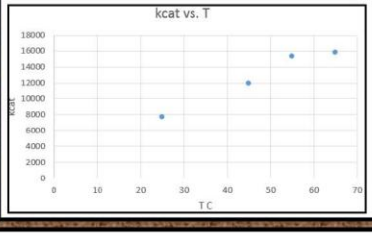


K_m=4,92±4,907mM $V_{máx}$=20,5±7,76mM/min
K_{cat}=102500min-1 **Catalytic efficiency**=41000mM^{-1}min^{-1}

Studies on the effect of temperature

The optimized essay was tested for different temperatures to study the variation of kinetic parameters:



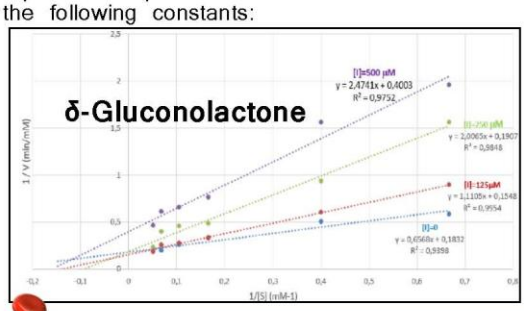


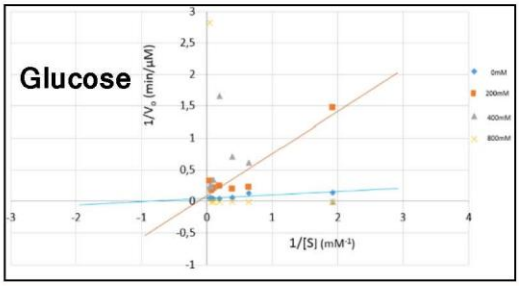
The studies yielded the following data:

E_a=15260J
Q_{10}=1,412

Inhibition studies

They were made with **δ-gluconolactone,** for inhibition via a transition estate analogue and **glucose** for inhibition via product. For every assay four different concentrations of inhibitor were used, in ideal reaction conditions. From the Lineweaver-Bruk plot and Dixon replot a competitive inhibition its observed for both species, with the following constants:



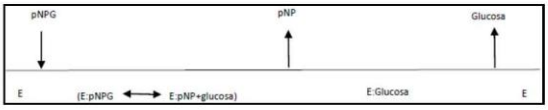


K_{EI} (glucose)= 150mM

K_{EI} (δ-gluconolactone)= 100 μM

Conclusion

The kinetic parameters obtained seem coherent, compared to other β-glucosidases. The enzyme displays its maximum activity at 60ºC. The competitive inhibition exerted by glucose suggests an ordered, tertiary-complex mechanism, detailed in the Cleland diagram. As δ-gluconolactone acts like a transition state analogue, we suggest a two-step catalysis, involving a covalent intermediate. Still, more experimentation is required, using p-NP as inhibitor, in order to demonstrate the proposed ordered mechanism.



KINETIC CHARACTERIZATION OF BETA GLUCOSIDASE OF ALMOND

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INTRODUCTION: Beta-glucosidases (b-D-glucopyranoside glucohydrolases, E.C. 3.2.1.21) **are enzymes that hydrolyze glycosidic bonds** to release nonreducing terminal glucosyl residues from glycosides and oligosaccharides. These enzymes are **found in all domains of living organisms**, in which they play a variety of functions (biomass conversion in microorganisms, breakdown of glycolipids and exogenous glucosides in animals, and lignification, catabolism of cell wall oligosaccharides, defense, phytohormone conjugate activation, and scent release in plants**). They have been classified into glycoside hydrolase (GH) families based on their amino acid sequences**. GH1, GH5, and GH30 b-glucosidases fall in GH Clan A, which consists of proteins with (b/a)8-barrel structures. In contrast, the active site of GH3 enzymes comprises two domains, while GH9 enzymes have (a/a)6 barrel structures.

The aim of this study is the kinetic characterization of almond β-glicosidase which would lead to propose a model for its catalytic mechanism.

METHODS: It was first standardized β-glucosidase optimal essay conditions, and then determined its kinetic parameters for pNPG, the effect of temperature on the catalysis, and studies of inhibition by product (glucose) and transition state analogue (δ-Gluconolactone). The general essay was proposed on figure 1. AMPLIAR MÉTODOS?

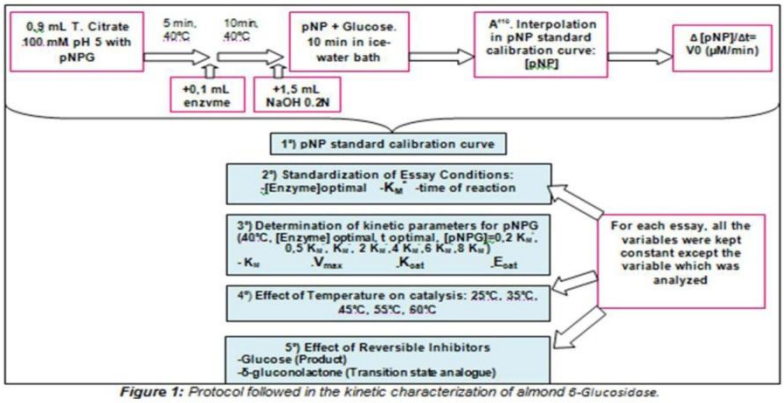

Figure 1: *Protocol followed in the kinetic characterization of almond δ-Glucosidase.*

RESULTS:

·Standard assay conditions: Firstly, a PNP straight pattern was made (Figure 2). Secondly, have been calculated standard assay conditions: optimal concentration of enzime (Figure 3), Km appearance calculate (Figure 4), linearity over time (Figure 5), and optimal temperature (Figure 6).

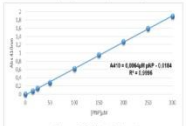
Figure 2: PNP straight pattern

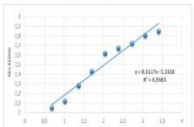
Figure 3: Optimal concentration of enzime

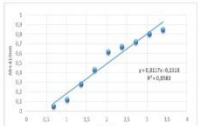
Figure 3: Optimal concentration of enzime

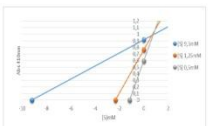
Figure 4: Representation of Eisenthal y Cornish-Bowden

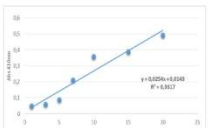
Figure 5: Linearity over time

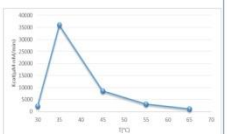
Figure 6: Optimal temperature

·Kinetic parameters for pNPG: Kinetic parametres are calculated by diferent representations (figure 7-10) by *"Hiperbolic regresión"* program.

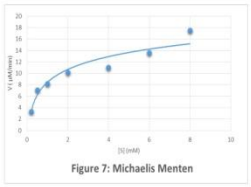
Figure 7: Michaelis Menten

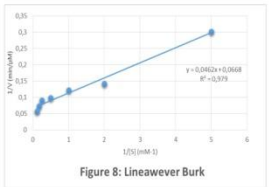
Figure 8: Lineawever Burk

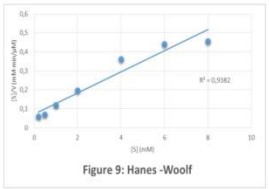
Figure 9: Hanes -Woolf

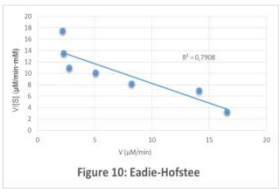
Figure 10: Eadie-Hofstee

·Studies of Inhibition by glucose and δ-gluconolactone. The study of inhibition takes place in increasing concentrations of inhibitor. Obtaining a Michalis Menten representation (Figure 11 and 16) and reverse dual representations (figure 12 and 14). The KEI of glucose and δ-gluconolactone was calculated by a secondary representation (figure 13 and 15). Finally, a Cleveland scheme was developed.(figure 17)

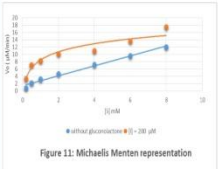
Figure 11: Michaelis Menten representation

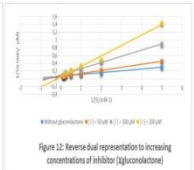
Figure 12: Reverse dual representation to increasing concentrations of inhibitor (Xgluconolactone)

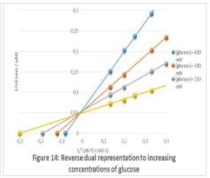
Figure 14: Reverse dual representation to increasing concentrations of glucose

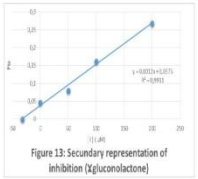
Figure 13: Secundary representation of inhibition (Xgluconolactone)

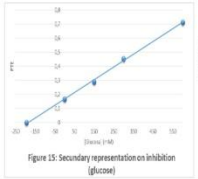
Figure 15: Secundary representation on inhibition (glucose)

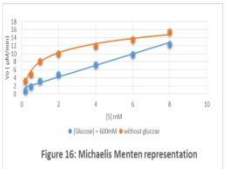
Figure 16: Michaelis Menten representation

CONCLUSIONS

•Standard assay conditions : 5 min of time of reaction, 2 nM of enzyme and 0,8 mM of apparent K_M (K_M^*)

• Kinetic parameters for pNPG : V_{MAX}=17,16 µM/min, K_M=1mM, K_{CAT}=8,58 min^{-1}, E_{CAT}=9,86 $mM^{-1}min^{-1}$

•Temperature assays: the maximum of activity was at 35ºC, Q_{10} around 7, and activation energy of 597,77 J/mol.

•Studies of Inhibition by glucose and δ-gluconolactone: both are competitor inhibitors, with KEI of 112 mM and 0,032 mM, respectively (so that, the second one is a better inhibitor).

•Kinetic mechanism: A ping-pong mechanism was proposed to this reaction, where PNP is the first product and the second is the glucose (Figure 16).

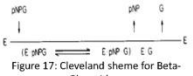

Figure 17: Cleveland sheme for Beta-Glucosidase

SUGGESTIONS

Further analysis will be needed to study the effects of variation of pH to develop the active center functionality. Also, could made a irreversible inhibition study, and reversible inhibition by its other product, Pnp

BIBLIOGRAPHY

- Masaru M, Sasaki J, Murao S. Studies on β-glucosidase from soybeans thathydrolyze daidzin and genistin: Isolation and characterization of an isozyme.Biosci Biotech Biochem 1995, 59: 1623–1627.
- Cleland, W.W. (1979) Methods Enzymol. 63, 500-513.
- Park, T. H.; Choi, K. W.; Park, C. S.; Lee, S. B.; Kang, H. Y.;Shon, K.J.; Park, J. S.; Cha, J. Substrate specificity and transglycosylationcatalyzed by a thermostable beta-glucosidase frommarine hyperthermophile Thermotoga neapolitana. Appl.
- Microbiol.Biotechnol. 2005, 69 (4), 411–422.
- Itohnashida, T.; Hiraiwa, M.; Uda, Y., Purification and properties of beta-D-glucosidase(linamarase) from the butter bean, Phaseolus lunatus. Journal of Biochemistry 1987,101, 847-854.
- Daniels LB, Coyle PJ, Chiao YB, Glew RH, Labow RS (1981)Purification and characterization of a cytosolic broad specificitybeta-glucosidase from human liver. J Biol Chem 256:13004–13013